

FORM PTO-1390 U S DEPARTMENT OF COMMERCE PATENT AND  
TRADEMARK OFFICE (REV 5-93)

ATTORNEY'S DOCKET NUMBER

0652.1830000/RWE/BEC

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. § 371**

U S APPLICATION NO (IF KNOWN SEE 37 C.F.R. § 1.5)

(to be assigned)

09/202984

INTERNATIONAL APPLICATION NO

PCT/EP97/03329

INTERNATIONAL FILING DATE

25 June 1997

PRIORITY DATE CLAIMED

28 June 1996

TITLE OF INVENTION

PROCESS FOR COMPARATIVE SCREENING OF SUBSTANCES HAVING A PHARMACOLOGICAL  
ACTIVITY

APPLICANT(S) FOR DO/EO/US

CZERNILOFSKY, Armin, Peter; VON RÜDEN, Thomas; HIMMLER, Adolf; LOEBER, Gerhard;  
METZ, Thomas; SCHNITZER, Renate; SPEVAK, Walter; STRATOWA, Christian, TONTSCH, Ulrike,  
WEYER-CZERNILOFSKY, Ulrike; WICHE-CASTANON, Maria, Josefa

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371
3. ☒ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau)
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 C.F.R. § 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A change of power of attorney and/or address letter
16. ☒ Other items or information:
  - a. Copy of the International Search Report for PCT/EP97/03329
  - b. Authorization To Treat A Reply As Incorporating An Extension Of Time Under 37 C.F.R. § 1.136(a)(3) (in duplicate).

03 Nov 1998 10:23 DEC 1998

U.S. APPLICATION NO (if known see 37 C.F.R. 1.50) (to be assigned)		INTERNATIONAL APPLICATION NO PCT/EP97/03329		ATTORNEY'S DOCKET NUMBER 0652 1830000/RWE/BEC	
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17. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>	<b>PTO USE ONLY</b>
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO . . . . . \$840.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) \$670.00					
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . . \$760.00					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . . \$970.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) . . . . . \$96.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT</b> =				\$ 840.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e))				\$ 130.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	28 - 20 =	8	X \$18.00	\$ 144.00	
Independent Claims	1 - 3 =	0	X \$78.00	\$ -0-	
Multiple dependent claim(s) (if applicable)			+ \$260.00	\$ -0-	
<b>TOTAL OF ABOVE CALCULATIONS</b> =				\$ 1,114.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must be filed. (Note 37 CFR 1.9, 1.27, 1.28)				\$ -0-	
<b>SUBTOTAL</b> =				\$ 1,114.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$ -0-	
<b>TOTAL NATIONAL FEE</b> =				\$ 1,114.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover (37 CFR 3.28, 3.31) \$40.00 per property				\$ -0-	
<b>TOTAL FEES ENCLOSED</b> =				\$ 1,114.00	
				<b>Amount to be:</b>	\$
				<b>refunded</b>	
				<b>charged</b>	\$

a. ☒ A check in the amount of \$ 1,114.00 to cover the above fees is enclosed

b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO: STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW, Suite 600 Washington, D.C. 20005-3934	<u>Robert W. Esmond</u> Signature Robert W Esmond Type Name 32.893 Registration Number	<u>Dec. 23, 1998</u> Date
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Czernilofsky *et al.*

Appl. No. (U.S. National Phase of  
PCT/EP97/03329; U.S. Appl.  
No. to be assigned)

Int'l Filing Date: June 25, 1997

For: **Process for Comparative Screening  
of Substances Having a  
Pharmacological Activity**

Art Unit: (to be assigned)

Examiner: (to be assigned)

Atty Docket: 0652.1830000/RWE/BEC

**Preliminary Amendment**

Assistant Commissioner for Patents

**Box PCT**

Washington, D.C. 20231

Sir:

Prior to examination on the merits of the above-identified patent application,  
please amend the application as follows:

***In the Specification:***

At page 1, line 1, before "Process for comparative screening of substances  
having a," please insert --Title of the Invention--;

line 6, please insert -- Field of the Invention--: and

line 9, please insert -- Background of the Invention --.

At page 3, line 32, please insert -- Summary of the Invention --.

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At page 4, line 14, please insert -- Detailed Description of the Invention --.

At page 25, line 19, please insert -- Examples --.

***In the Claims:***

Please add the following new claim:

--28. The processes according to claim 2, wherein said substances are applied in several dilutions.--

Please amend claims 1-3, 5-8, 17, 21 and 27 as follows:

In claim 1, at page 53, lines 16 and 26, please delete "and/".

In claim 2, at page 54, lines 3-4, please delete "optionally in several dilutions,".

In claim 3, at page 54, line 9, please delete "or 2".

In claim 6, at page 54, line 19, please delete "one of claims 1 to 4" and insert --claim 1-- therefor.

In claims 5, 7, 8 and 27, please delete "one of the preceding claims" and insert --claim 1-- therefor.

In claims 17 and 21, please delete "one of claims 1 to 7" and insert --claim 1-- therefor.

### ***Remarks***

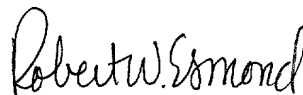
Upon entry of the foregoing amendments, claims 1-28 are pending in the application. Claim 28 is sought to be added. Claim 28 finds support, *inter alia*, at page 6, line 14, and in original claim 2, of the application as filed. Claims 2, 3, 5-8, 17, 21 and 27 have been amended to eliminate multiple dependencies.

The specification has been amended to include section headings, thus conforming the application to the preferred layout for a patent application as suggested in M.P.E.P. § 601.

Applicants respectfully submit that no new matter has been added by way of the above amendments. Applicants request that these amendments be entered into the application prior to examination.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Robert W. Esmond  
Attorney for Applicants  
Registration No. 32,893

Date: Dec. 23, 1998

1100 New York Avenue, NW  
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09/202984

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Czernilofsky *et al.*

Appl. No. (U.S. National Phase of  
PCT/EP97/03329; U.S.  
Appl. No. 09/202,984)

Filed: (Int'l Filing Date: June 25, 1997)

For: **Process for Comparative  
Screening of Substances Having a  
Pharmacological Activity**

Art Unit: (To be assigned)

Examiner: (To be assigned)

Atty. Docket: 0652.1830000/RWE/BEC

**Response to Notification of Missing Requirements**

Assistant Commissioner for Patents  
**Box PCT**  
Washington, D.C. 20231

Sir:

The Notification of Missing Requirements dated July 13, 1999, indicates that the current oath or declaration of the inventors does not comply with 37 C.F.R. § 1.497(a) and (b). The appended Notification of a Defective Oath or Declaration states that the perceived reason for non-compliance is that the oath or declaration does not identify the citizenship of each inventor. The Notification contains a marginal note requesting clarification of the fact that an inventor, Maria Josefa Wiche-Castanon, has designated two countries of citizenship.

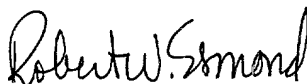
In response, Applicants advise that Maria Josefa Wiche-Castanon is a dual national. She is simultaneously a citizen of Spain and Austria. The requested clarification having been

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provided, Applicants respectfully submit that the declaration does comply with 37 C.F.R. § 1.497(a) and (b) and that the application is now fully in condition for entry into the national stage in the United States.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Robert W. Esmond  
Attorney for Applicants  
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Date: Aug 12, 1999

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Process for comparative screening of substances having a pharmacological activity

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The invention relates to a process for screening substances having a pharmacological activity.

- 10 Screening processes with a high throughput rate ("High Throughput Screening") and related screening technologies have become the most important processes for discovering new active substances in the pharmaceutical industry. Generally, processes of this kind are defined by the fact
- 15 that a large number of substances originating from pure chemical banks, natural products or from combinatorial chemistry are tested in specific biological assays, designed in terms of the desired effect of the drug, in a most cost-effective manner and in the shortest possible
- 20 time. Conventional types of assay, e.g. receptor binding or enzyme substrate assays, have already been modified relatively easily to operate by the high throughput format, which has been made necessary by the progress made with the apparatus, e.g. the methods of handling on a
- 25 liquid basis, and the detection methods, combined with robot systems.

- Assay systems based on living cells have hitherto been used less frequently in high throughput screens,
- 30 particularly because of the difficulties encountered on standardisation, the problem of interpreting the results from the primary screening and the unsatisfactory nature of the equipment in overcoming the demands of cellular systems and long incubation times. Another reason why the
- 35 use of cellular assays for high throughput screening has hitherto been regarded as being less attractive is in the

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large outlay involved in primary screening, i.e. the first screening process, to distinguish between toxic, non-specific or non-selective effects of the substance or to clarify the mechanisms of activity occurring in the cell when the cellular system responds to a substance, i.e. in the event of a "hit".

With the aid of the highly developed techniques of molecular biology, biological target molecules the activity of which is supposed to modulate the drug to be identified can be incorporated in eukaryotic cells, particularly mammalian cell lines. In addition, there are various methods available for detecting the desired effect in the target cell, e.g. the production of a new protein as the result of the stimulation of a specific receptor, the activation or increase in measurable events coupled to the target mechanism, e.g. the expression of a reporter gene product such as luciferase or Green Fluorescent Protein (GFP) or influencing key events which occur in the cell after receptor activation, e.g. apoptosis.

The characteristics of a good cell-based assay are as follows:

- a) The "signal/noise" ratio, i.e. the ratio of maximum change and normal level of the measuring signal, has to be a favourable one, i.e. the background signal should be low enough to detect induction of the event to be measured with high sensitivity but at the same time high enough to be able to determine the detection limits with respect to the negative controls.
- b) The signals measured must be reproducible and stable over a certain period of time.
- c). The test cells must be relatively easy to culture and resistant to treatment in the assay.
- d) The biological target molecule introduced into the cells and the regulation mechanisms coupled thereto must be stable over

a fairly long period (several months). e) The properties of the cell must remain the same over a fairly long period of time; i.e. the cell lines used must also be stable *per se*.

5

Satisfying these requirements constitutes the basis for a screening system the chief advantage of which is that living cells prepare a substrate which provides the substances to be tested with the target molecule which they are supposed to, in the last analysis, modulate in the living organism, being potential therapeutic drugs, in the correct biological environment. One example of a test substrate consists, for example, of cells of a cell line which contain a cloned receptor and the signal transmission pathway associated therewith, coupled to a suitable reporter system.

High throughput strategies are highly efficient if the information obtained in the primary screening can be maximised. At the same time, this means that the outlay connected with the primary screening which consists in qualitatively differentiating the "hits" obtained must be kept to an absolute minimum. With the screening throughput rates now achievable, of the order of a million substances per annum, a "hit" rate of more than 1% results in unacceptable expenditure. Typical hit quotas of more than 1% and the complex secondary assays connected with them mean that, in spite of the theoretical advantages of cellular assays, the user will frequently fall back on alternative screening formats such as receptor binding or enzyme substrate assays.

The objective underlying the present invention is to maximise the theoretical advantages of cellular assays and provide a process which does away with the disadvantages

that occur in cellular assays of the high throughput format.

- 5 The concept of "parallel screening" is generally applied to the logistic mechanism in screening whereby a number of different assays or assay formats are carried out with the same arrangement of equipment under the control of a robot.
- 10 The present invention was based on the idea of further developing this approach for comparative screening, by simultaneously charging a number of different cellular assay formats from the same supply of substance.
- 15 The present invention relates to a process for determining the pharmacological activity of a substance on the activity of different biological target molecules by applying the substance to test cells which contain one or more biological target molecules and determining the
- 20 effect of the substance on the activity of the target molecules. The process is characterised in that a defined quantity of a test substance, in one operation, is
- 25 a) applied to test cells with the same basic biological constitution which differ in that they contain one or more different biological target molecules; and/or
- 30 b) is applied to test cells which contain one or more biological target molecules, the cells differing in that they have different basic biological constitutions, and in that
- 35 i) the effect of the substance on the biological target molecule or molecules is measured by means of a detection system coupled to the activation of the target molecule; and/or

ii) the effect of the substance on different regulatory mechanisms triggered by the activation of the target molecule is determined by measuring the effect using a plurality of detection systems each coupled to the different regulatory mechanisms,

and in that the effects of the test substance on the different test cells or the effects determined by different detection methods are compared directly with one another.

The test cells used in the process according to the invention and containing one or more specific biological target molecules constitute a defined cellular substrate onto which the test substance in question is applied. The substrate (in case a) cells with identical biological background and different target molecules; in case b) cells with different biological background (basic constitution) and identical target molecule) generally consists of a number (up to about  $10^5$ ) of identical cells each located in a well of a culture plate. If desired, single cells may also constitute the substrate and be acted upon by the substance if the detection system is sensitive enough, e.g. if, in the event of measuring optical signals, the conditions in the apparatus are able to amplify the signals accordingly by physical methods.

The main feature of the invention consists in the fact that the same substance is applied to a number of different cellular substrates in one operation. Each substrate represents an individual assay type. If different detection systems are used to investigate the effect of the test substance on different regulatory mechanisms of the same target molecule, there are different assay formats based on the same substrate.

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target molecule in question endogenously. However, it is preferable to use cells which do not express the target molecules in question at all or express them only slightly. The cells are transformed by conventional  
5 transfection methods with the DNA coding for the biological target molecule, e.g. with receptor DNA (cf. for example Potter et al. 1984; Felgner et al., 1987); the electroporation, calcium phosphate precipitation or lipofection methods are preferred.

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The cells are used on the one hand to produce test cells by forming the starting substrate for receiving the DNA coding for the target molecule, and on the other hand they are used as control cells to check whether a signal  
15 measured by a detecting system can actually be attributed to the effect of the test substance. If the substance generates a signal in the test cell but no signal in the starting cell used as the control cell, the effect detected by means of the signal can be attributed to the  
20 test substance. If the control cell also emits a signal, the substance (also) influences a cellular process which is independent of the activation of the target molecule; the control measurement corresponding to this signal must be taken into account when evaluating the measurement.

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With a view to carrying out the method of measurement used as simply as possible, cells are used which satisfy the prerequisites for this purpose. For test cells in which proliferation or apoptosis is measured, it is possible to  
30 use both adherent cells and cells which grow in suspension and the survival of which in culture is strictly dependent on the presence of certain growth factors. If these growth factors are removed, the cells stop growing or initiate apoptosis within a few hours. For test cells  
35 based on the measurement of gene expression, it is preferable to use cells which adhere well, e.g.

fibroblasts, epithelial cells, endothelial cells, etc., as this method of measurement requires a washing step.

5 If according to embodiment a) of the process according to the invention cells are used having the same basic biological constitution, these are cells of the same type, i.e. cells isolated from a certain tissue and defined *inter alia* by molecular-biological, morphological, biochemical and immunological parameters. Preferably, the  
10 test cells used are clonal cells, generally cells of a permanent cell line. These cells originate from a single original cell and have an identical genotype.

15 For the embodiment of the invention according to b) cells having a different basic biological constitution are, by definition, not only cells of different types but also cells of the same type but with a different state of differentiation or activation. In this way, for example, the effect of a test substance on normal cells can be  
20 compared with its effect on tumour cells coming from the same tissue, or the effect of the substance on cells of an established cell line can be compared with the effect on primary cells of the same origin, thus making it possible to assess the predictive ability of screening based on  
25 permanent cell lines.

Biological target molecules are those molecules whose biological activity is supposed to be influenced by the test substance in order to block an unwanted reaction of  
30 the cell which is responsible for a pathological effect, this reaction resulting from this activity.

Theoretically, target molecules may be any of the proteins or fragments or mutants thereof which naturally occur in  
35 mammalian cells, particularly human cells, which are constitutively active or the activation of which by an

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extracellular signal (the natural ligand) brings about a reaction of the cell which can be measured directly or indirectly in any form. The influence on this reaction which occurs upon inhibition by a test substance is thus also measurable, making it possible to obtain automatic evaluation when the process is used as a robot-controlled high throughput process.

Biological target molecules are components of various cellular, *inter alia* metabolic or receptor-coupled signal transmission pathways; in theory, these components may originate from any section of the signal transmission cascade.

Examples of biological target molecules are representatives of the major group of receptors, particularly receptors for growth and differentiating factors including their subtypes. Examples of receptors include receptor tyrosine kinases (EGF receptor, PDGF receptor, etc.); serine/threonine kinases such as the TGF- $\beta$  receptor, integrin receptors; receptors of class LIF, oncostatin M, CNTF, etc. (gp130); receptor phosphatases; receptors for cytokines (interleukins, interferons, etc.) and G-protein-coupled receptors which are able to couple to the phospholipase C-signal transmission pathway or the adenylate cyclase signal transmission pathway, e.g. neuropeptide Y receptors (also PYY, NPY) or neurokinin receptors (NK<sub>1,2,3</sub>).

A process for screening substances with a modulating effect on receptor-dependent cellular signal transmission pathways and its application to various receptors is described for example in WO 93/11257, to the contents of which reference is hereby made. The principle of the process is based on the use of test cells which contain a reporter gene under the control of a regulatory sequence

which responds to a change in the concentration of a messenger substance of a receptor-dependent signal transmission pathway (sensor DNA) and the receptor DNA in question. If the test substance is applied to the test  
5 cells, the effect of the test substance on the target molecule, i.e. the receptor in question or an element of the signal transmission pathway coupled to this receptor, takes the form of a change in the expression of the reporter gene. According to embodiment a) in conjunction  
10 with i) of the process according to the invention, for comparative screening on the basis of this assay principle, identical starting cells are transformed with identical reporter gene construct (sensor DNA) and with different receptor DNA, thus making it possible to make a  
15 direct comparison of the effect of the test substance on the activation of signal transmission pathways of different receptors or receptor subtypes.

According to alternative b) in conjunction with i), cells  
20 of different types are transformed with identical receptor DNA and identical sensor DNA in order to investigate the effect of the test substance on the signal transmission pathway coupled to the receptor in question in various cell types. This is particularly interesting when  
25 searching for a drug which has selectivity for a certain cell type, i.e. when the substance is intended to influence the signal transmission pathway only in a certain type of cell but not in other types of cell, e.g. the signal transmission pathway of the IL-5 receptor which  
30 is expressed on eosinophils and certain T-cells but not on other cells.

The activation of a receptor or receptor subtype can, as the result of complex intracellular interactions, initiate  
35 the activation of more than one signal transmission pathway/effector system, e.g. if a G-protein-coupled

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receptor is able to interact with more than G-protein variant. For example, the angiotensin AT<sub>1</sub>-receptor couples with the phospholipase C and adenylate cyclase signal transmission pathway, whereas the AT<sub>2</sub>-receptor couples with the effector system of cyclic GMP. Another example is the prostanoid receptor EP<sub>3</sub>, which couples to the phospholipase C and adenylate cyclase signal transmission pathway, whereas other prostanoid receptors couple only to the adenylate cyclase signal transmission pathway or, in one case, a nuclear receptor (PPAR $\gamma$ ) is activated directly. If the cause of pathological changes in the cell lies in disruption of these interactions, it may be useful to discover drugs which act specifically on only one of the effector systems. In this case, the embodiment of the process according to a) in conjunction with ii) can be used. If for example one signal transmission pathway is the phospholipase C-effector system which when activated causes the messengers inositol-1,4,5-triphosphate (IP<sub>3</sub>) and diacyl glycerol (DAG) to be formed, and the other is the adenylate cyclase effector system in which the messenger substance cAMP is formed, two test cells transformed with the same receptor can be used, which differ in the sensor DNA, one of them responding to the IP<sub>3</sub>/DAG concentration and the other responding to the cAMP concentration. Examples of suitable sensor DNA constructs can be found in WO 93/11257. The invention makes it possible in this case to state directly in one screening operation whether a substance has specificity for only one signal transmission pathway.

Alternatives ai) and aii) may also be combined in one screening process.

Other examples of target molecules are intracellular components of signal transmission pathways, e.g. protein

kinases (Src, Raf, MAPK, JAK), adaptor molecules (GRB, Sos) or other elements of signal transmission pathways such as Ras.

5 Target molecules may also be components located further downstream in the signal transmission pathway, such as elements of inducible gene expression (ligand-regulated transcription control), particularly transcription factors, e.g. Myc, Fos, Myb, NF- $\kappa$ B, AP-1, STAT, Ets-  
10 proteins and/or the co-factors thereof, or molecules which participate in cell apoptosis (Bcl-2, p53 mutants or the proteases which regulate apoptosis), as well as kinases, phosphatases or GTPases. The target molecules may also be intracellular hormone receptors such as steroid receptors  
15 or retinoid receptors. The skilled person will be familiar with such elements; an overview of inducible gene expression and the components involved therein can be found in the relevant textbooks, e.g. "Inducible Gene Expression", 1995.

20 The target molecules may be identical to the proteins naturally occurring in the cell; however versions of these proteins which have been mutated in various sections or which have been changed, e.g. shortened, compared with the  
25 Wild type in certain sections may also be used. Thus, for example, it is possible to investigate the influence of a test substance on certain domains of the target molecule, e.g. on the transactivation domain or on a section of the protein which is responsible for interaction with a co-  
30 factor.

The effect of a test substance on the biological target molecule is perceived by the cell as a signal that leads to different reactions of the cell depending on the cell  
35 type. Such reactions, e.g. a change in the intracellular calcium level, phosphorylation or dephosphorylation of

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that test cells from which the growth factor has been withdrawn and test cells incubated in the presence of growth factor have a significant difference in the number of cells in this length of time.

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Another method of detection preferably used within the scope of the present invention is based on measuring the expression of reporter genes.

- 10 A reporter gene is defined by the fact that its expression product can be detected and quantified by the measurement of a signal the magnitude of which is proportional to its concentration. Within the scope of the present invention, any reporter genes whose expression can be measured with a  
15 high degree of sensitivity in an automated assay may be used.

- Examples of reporter genes are the alkaline phosphatase gene and the  $\beta$ -galactosidase and  $\beta$ -glucuronidase gene; the  
20 reactions catalysed by these enzymes are monitored using established fluorescence assays (Wieland et al., 1985; Kricka, 1988), and also chloramphenicol-acetyltransferase (CAT; Hartmann, 1991).

- 25 A reporter gene which is preferred within the scope of the present invention is the gene coding for Photinus pyralis luciferase (firefly luciferase) (De Wet et al., 1987). This enzyme has the advantages that with its substrate luciferin and with the addition of ATP it produces  
30 bioluminescence in a high yield, which can be measured by established automatable methods, and this enzyme is not produced endogenously by mammalian cells. Moreover, luciferase has a relatively short half-life *in vivo* and is also not toxic in high concentrations (Hartmann, 1991;  
35 Brasier et al., 1989). Measuring the activity of firefly luciferase by bioluminescence is one of the most sensitive

methods of determining an enzyme. For this reason and because of the absence of luciferase activity in normal mammalian cells, this enzyme is particularly suitable as a reporter gene (Subramani and DeLuca, 1987). Another  
5 preferred report gene is Green Fluorescent Protein (GFP; Inouye and Tsuji, 1994; Chalfie et al., 1994).

Reporter genes may be used *inter alia* to detect the transcription of genes triggered by the effect of the test  
10 substance:

The stimulation of a number of different signal transmission pathways ends in the transcription of a group of genes, e.g. the so-called "immediate early genes" such  
15 as c-fos, c-jun, c-myc. Thus, by measuring the expression of these genes, it is possible to demonstrate the effect of test substances on the activation or inhibition of various signal transmission pathways.

20 Direct measurement of gene expression, e.g. using antibodies against the expression products, is technically laborious and cannot generally be used for automatic screening. By contrast, the expression of selected genes can be determined indirectly by a relatively simple  
25 method. The effect of the test substance on the target molecule is determined by measuring the expression of a reporter gene (e.g. the luciferase gene) which is under the control of the regulatory sequence of the original gene (e.g. c-fos), instead of measuring the expression of  
30 the original gene, i.e. the gene which is naturally expressed at the end of the intracellular signal cascade triggered by the activation of the target molecule. The expression levels for the reporter gene are directly proportional to the effect of the test substance on the  
35 target molecule. A screening process of this kind based on transcription modulation which is suitable for use in

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the high throughput format is described in WO 92/13063, the contents of which are hereby referred to.

5 The various detection systems used within the scope of the present invention either simultaneously in one step or consecutively in subsequent screening steps may differ on the one hand in the detection principle itself (e.g. reporter gene expression as against apoptosis) and/or in the design of the detection system. This makes it  
10 possible to give differential statements as to the effect of a test substance on different regulatory mechanisms which result in different, in some cases pathological, effects (e.g. the use of different sensor DNA molecules which respond to different effector systems or contain  
15 promoters of different target genes). Thus, the process according to the invention opens up the possibility of differentiation in terms of the desired prevention of a certain unwanted biological effect. If for example one wishes to find a substance which inhibits the  
20 proliferation of a certain cell type but not differentiation, and it is known that these two reactions are evoked in the same cell by activation of different regulatory mechanisms of the same target molecule, the effect of the test substance can be assigned to one of the  
25 two signal transmission pathways by a suitable assay design. For this purpose, for example, the effect of the substance on the same cell type can be determined in a comparative screening with two different detection systems each of which responds to a different regulatory  
30 mechanism. If it is known that the activation of a certain regulatory system is correlated to a certain undesirable reaction in the cell, e.g. if the activation of a certain signal transmission pathway leads to apoptosis, the effect of the test substance on apoptosis  
35 can be determined not only directly by measuring apoptosis but also indirectly by means of a reporter gene system



coupled to this signal transmission pathway. In this case, the expression of the reporter gene product as a consequence of the effect of the test substance bears witness to the fact that the substance would influence  
5 apoptosis for the selected cell type.

The process according to the invention also makes it possible to arrive rapidly and efficiently at a pronouncement regarding a target molecule which is already  
10 present in cloned form but the function of which is wholly or partially unknown, e.g. a particular receptor subtype. If a test cell transformed with the receptor of unknown function is used, together with a series of test cells of identical basic biological construction which contain  
15 other receptors of known function, in an assay the detection system of which detects the influence of test substances on a biological activity either directly or indirectly via a reporter gene system, comparison of the effect of the test substances on the test cells containing  
20 various receptors makes it possible to comment on the function of the receptor whose function has hitherto been unknown.

Within the scope of the present invention, cells of a  
25 growth factor-dependent cell line were transformed *inter alia* with different functional biological target molecules the activation of which plays a part in tumour progression, and used as substrate in a High Throughput Screen. Each group of cellular test substrates consisting  
30 of cells transformed with one of the biological target molecules was acted upon, several times over, with individual substances from a series of test substances and the proliferation of the cells was measured. The target molecules used were the EGF (Epidermal Growth Factor)  
35 receptor, the HER2 receptor, the receptor (KDR) for the vascular endothelial cell growth factor (VEGF), the

MET-HGF (Hepatocyte Growth Factor) receptor and activated Ras proteins.

5 These target molecules were chosen on account of their significance as potential intervention sites in tumour therapies:

10 The EGF receptor (EGF-R) is credited with playing an important part in the formation of various tumours of epithelial origin. This type of receptor is frequently constitutively activated in tumour tissues, specifically by overexpression, gene amplification, autocrine stimulation of the mutations. In the light of this observation, EGF-R is a potentially important point of  
15 attack in tumour therapy. Apart from EGF-R-specific monoclonal antibodies and toxin-charged ligands, some selective inhibitors of EGF-R-associated tyrosinekinase have hitherto been described. In the search for new inhibitors of EGF-R, cellular test systems have been  
20 developed within the scope of the present invention based on FDC-P1 cells which have been genetically altered so as to express functional human EGF-R. Since the FDC-P1 cells are IL-3-dependent, the proliferation of these cells can be stimulated either by murine IL-3 or by EGF (which may  
25 replace IL-3 in cells which express the EGF receptor ectopically) (von Rüden, T. et al., 1988; Pierce, J. H. et al., 1988). If these cells are cultivated entirely without growth factor, the cells die within 24 hours (apoptosis). The same is true of cells incubated in the presence of EGF  
30 and selective inhibitors of EGF-R. The proliferation or apoptosis rate depends on the concentration of the inhibitor. In order to check for non-specific toxicity of the test substances the influence of the test substances on the IL-3-dependent proliferation can be measured. These  
35 cells are suitable for screening for selective inhibitors of EGF-R.

Like the EGF receptor, the HER2 receptor is connected with the formation of epithelial tumours. As with the EGF receptor test cell, in the tests according to the present invention a test cell was set up, based on the parental cell line FDC-P1 for the HER2 receptor. Since the HER2 receptor unlike the EGF receptor normally does not transduce any mitogenic signals, being a homodimer, for setting up the test cells an HER2 mutant was used wherein the amino acid valine in position 654 was replaced by the amino acid glutamic acid. This amino acid exchange corresponds to the mutation in the *neu*-oncogene in the rat (Weiner, et al., 1989; Suda, et al., 1990) and means that the HER2 receptor is constitutively active even without ligands and without the formation of heterodimers. Ectopic expression of the mutated receptor in FDC-P1 cells induces IL-3-independent proliferation. Like the EGF-R-test cell, the HER2-test cell is suitable for finding selective inhibitors of the receptor.

The HGF receptor (c-met) thus plays a part both in tumour formation and, as a result of the ubiquitous presence of its ligand HGF in the surrounding connective tissue, in tumour invasion and metastasis. The HGF receptor is frequently overexpressed and/or amplified in tumour tissues. Up till now, no selective inhibitors of HGF-R-associated tyrosinekinase have been described. Within the scope of the present invention, in the search for inhibitors of HGF-R on the one hand analogously to EGF-R a cellular test system has been developed based on FDC-P1 cells which have been genetically altered so as to express functional human HGF-R. These cells require either IL-3 or HGF in order to survive. On the other hand KB cells which express human HGF-R (and human EGF-R) are stably transfected with a reporter gene so that activation of the receptor with HGF (or EGF) causes induction of the

reporter gene product, i.e. luciferase, which is easy to measure.

5 An important prerequisite for tumour growth is the blood supply. This is ensured by the formation of new blood vessels in the tumour, neoangiogenesis. For this, in an oxygen deficiency tumour cells produce the messenger substance VEGF which, after binding to the corresponding receptor "KDR", which is a receptor-tyrosinekinase (RTK),  
10 induces the proliferation and migration of the endothelial cells towards the tumour. The blockade of neoangiogenesis by inhibiting the VEGF receptor KDR is therefore an important approach in tumour therapy. Analogously to the test cells for EGF-R and HER2 test cells were prepared for  
15 the VEGF receptor KDR. Expression and activation of this RTK leads to IL-3-independent proliferation and is therefore suitable for screening for selective inhibitors.

20 Mutations of the Ras proteins H-Ras, K-Ras and N-Ras, which lead to constitutive activation of Ras, are found in many human tumours (Bos, 1988; Kiaris and Spandidos, 1995). The occurrence of Ras-mutations is therefore regarded as an important step in tumour formation, and consequently Ras-mutants are deemed to be target molecules  
25 in the treatment of tumours. Various Ras-inhibitors, such as monoclonal antibodies (Furth et al., 1982), dominant-negative mutants (Stacey et al., 1991; Quilliam et al., 1994), antisense RNA or inhibitors of the Ras-farnesyltransferases (Kohl et al., 1993; Kohl et al.,  
30 1994; Kohl et al., 1995), have confirmed the accuracy of the therapeutic principle of using Ras-mutants as points of intervention in the treatment of tumours. The inhibitors currently available are of only limited suitability for therapeutic purposes, however, since Ras-specific inhibitors (antibodies, dominant-negative mutants  
35 or antisense RNA) under *in vivo* conditions cannot be

introduced into all tumour cells in sufficient concentrations, and on the other hand the farnesyltransferase-inhibitors are not selective for mutated Ras, but will also influence the posttranslational  
5 modification of normal Ras, as well as other cell proteins, such as e.g. lamin A and B, skeletal muscle phosphorylasekinase, and retina proteins such as transducin and rhodopsinkinase. Moreover, K-Ras, which is most frequently mutated in human tumours, is also a  
10 substrate for the posttranslational modification by geranylgeranyl-transferase, which is only inadequately blocked by the farnesyltransferase inhibitors (James et al., 1995; Lerner et al., 1995; Manne et al., 1995). The test cells developed within the scope of the present  
15 invention can be used to search for low-molecular inhibitors of the activated Ras proteins. Within the scope of the present invention FDC-P1 cells were also used as test cells for potential Ras-inhibitors. Ectopic expression of the activated Ras-mutants K-Ras<sup>Val12</sup> (McCoy et al., 1984) and H-Ras<sup>Val12</sup> (Reddy et al., 1982) induces  
20 IL-3-independent proliferation of the test cells (Fig. 1 and 2). The use of a Ras-inhibitor known from the literature, e.g. the farnesyltransferase inhibitor L-739.749 (2(S)-{2(S)-[2(R)-amino-3-mercapto]propylamino-  
25 3(S)-methyl}pentylloxy-3-phenylpropionylmethionine sulfonemethyl ester; Kohl et al., 1994)) demonstrated the dependency of the test cells on the presence of the activated Ras proteins (Fig. 1 and 2). The cells used within the scope of the present invention are suitable for  
30 searching for low-molecular Ras-inhibitors and can be used in a High Throughput Screen.

In order to check the specificity of potential Ras-inhibitors control cells may be used which have been made  
35 IL-3-independent by the expression of activated proteins provided after Ras in the Ras-signal transduction pathway

and thus render cell growth independent of Ras (e.g. the signal transmission pathway which runs via Raf, MEK and ERK (= MAP Kinase) or the one which runs via Rac and Rho - optionally in combination with a second signal such as e.g. c-myc). A cell line of this kind is FDC-P1/J2, prepared by infection with a retrovirus which brings about the expression of activated v-raf and v-myc (Rapp et al., 1985). In addition to the activity of the abovementioned target molecules, overexpression of the Bcl-2 protein is regarded as one of the causes of certain malignant diseases, such as e.g. leukaemias (Reed, 1994), and furthermore overexpression of this protein is also described in tumours of epithelial origin (McDonnell, et al., 1992; Pezzella, et al., 1993; Lu, et al., 1993). There is a clear connection between Bcl-2 levels and resistance to chemotherapeutics (Kitada, et al., 1994; Miyashita and Reed, 1992). The molecular mechanism of activity of Bcl-2 is the prevention of programmed cell death (Reed, 1994). Bcl-2 thus counts as a potentially important site for attack in the treatment of certain leukaemias and solid tumours. FDC-P1 Test cells which overexpress Bcl-2 may be used for screening for low-molecular inhibitors of Bcl-2 protein, for example; these cells survive even in the absence of IL-3.

Substance "hits" obtained with the aid of a screen based on these target molecules can be classified differentially in a single step, even during the first screening, according to their activity on the target molecules which are involved in the tumour progression as the result of various mechanisms. Thus it is possible to find substances for the therapy with a very specific activity.

Apart from the cells designated FDC-P1 used in embodiment 1 of the present invention, the preparation of which was

described by Dexter, T. M. et al., 1980, in which screening was carried out for potential tumour therapy drugs, other growth factor-dependent cells may be used for screening based on the measurement of proliferation as a  
5 detection system; e.g. the cell line 32D described by Pierce, J. H. et al., 1988, or the cell lines designated BAF-B03 or DA-1 described by Shibuya, H. et al., 1992, or Alexander, W. S. et al., 1991.

10 The assay system may be constructed as follows, for example:  
The cells are placed in 96-well microtitre plates in the presence of the corresponding growth factors. Then, e.g. using a pipetting robot such as Tecan RSP5052, dilutions  
15 of the substances to be tested are added and the test cells are incubated for a certain length of time under conventional cell culture conditions. Then the cells are mixed with assay reagent, e.g. the MTS-based "Cell titer non-radioactive proliferation assay" (Promega) and after  
20 further brief incubation, measured in a microtitre plate photometer (e.g. SLT Spectra Shell). The incubation period must be such that test cells deprived of growth factor and test cells which are in the presence of growth factor exhibit a distinct difference in the number of cells  
25 during this period. Substances which specifically inhibit a receptor tyrosinekinase (RTK) should not have any effect in cell lines which are dependent on a different RTK.

Another assay used within the scope of the present  
30 invention is based on the human epithelial cell line KB4 into which the luciferase gene has been introduced under the control of the human fos-promoter. These cells endogenously express the human EGF receptor, the human HGF receptor as well as some other receptor tyrosinekinases.  
35 Treatment of the cells after removal of serum with a ligand of these receptor tyrosinekinases leads to

activation of the fos promoter and expression of the luciferase gene, the activity of which is proportional to the strength of the activation signals over a certain range. A corresponding test system for High Throughput  
5 Screening can be set up as follows: the cells described above are placed in serum-reduced medium in 96-well microtitre plates. Then dilutions of the substances to be tested are added using a pipetting robot (e.g. Tecan RSP5052) and the luciferase gene under fos control is  
10 induced with the corresponding growth factor. The test cells are incubated for a certain length of time under conventional cell culture conditions. Then the cells are washed with a buffer solution and mixed with an assay reagent which lyses the cells and contains the components  
15 luciferin and ATP needed for the luciferase reaction. After brief incubation the luciferase signal is measured in a microtitre plate luminometer (SLT Spectra Shell; Wallac Microbeta). A specific RTK inhibitor reduces the luciferase signal in the corresponding test cell, but has  
20 no influence on the c-fos promoter activity in other test cells.

#### List of Figures

25

Fig.1: Inhibition of H-ras<sup>Val12</sup> dependent proliferation (viability) by the farnesyltransferase inhibitor L-739.749 in IL-3-free medium

30

Fig.2: Inhibition of H-ras<sup>Val12</sup> dependent proliferation (DNA synthesis) by farnesyltransferase inhibitor L-739.749 in IL-3-free medium



Fig.3: Detection of the overexpression of H-ras<sup>Val12</sup> in FHRV12cc21 cells and inhibition of Ras-farnesylation by L-739.749

5 Fig.4: Detection of HGF-dependent proliferation or viability by measuring the incorporation or absorption of <sup>3</sup>H-thymidine for various concentrations of recombinant HGF

10 Fig.5: Dosage-dependent induction of luciferase activity in KB-B4 cells by HGF or EGF

Fig.6: Selective inhibition of EGF-induced, but not HGF-induced luciferase activity by an EGF-R inhibitor

15

Fig.7: Selective inhibition of the proliferation of an EGF-dependent cell line by an EGF-R inhibitor

20

#### Example 1

Proliferation assay of the High Throughput Format for screening substances with an anti-tumour activity

25

a) Preparation of a test cell for activated H-Ras<sup>Val12</sup>

i) Expression vector for human H-Ras<sup>Val12</sup>

30 Suitable vectors for the ectopic expression of activated human Ras proteins are retroviral vectors such as e.g. pGD (Daley et al., 1990), pLXSN (Miller and Rosman, 1989), or expression plasmids with strong eukaryotic or viral promoters such as e.g. SV40 or cytomegalovirus (CMV),  
35 under whose transcriptional control the cDNA or the gene for an activated Ras protein are placed. The test cell was

prepared using the expression vector pGDV12ras (Tanaka et al., 1994) which was brought under the control of the murine retroviral LTR by cloning the oncogenic form of human cDNA for c-H-ras<sup>Val12</sup> into the EcoRI site of the retroviral vector pGD (Daley et al., 1990). The pGD vector additionally contains the neomycin-phosphotransferase gene under the control of a thymidinekinase promoter from plasmid pMC1neo (Stratagene), which, with neomycin analogues (G-418), makes it possible to select cells which have the expression construct stably integrated in their genome.

#### ii) Transfection

FDC-P1 cells (Dexter et al., 1980) were placed in IMDM medium (BioWhittaker), combined with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol and 1% IL-3 and incubated at 37°C in 5% CO<sub>2</sub>. 10<sup>7</sup> cells per transfection were centrifuged off for 5 min at 1200 rpm at ambient temperature (Heraeus Minifuge), washed once with serumfree RPMI-1640 medium, and suspended in 1 ml serumfree medium. 800  $\mu$ l of this cell suspension were mixed with 20  $\mu$ g of plasmid pGDV12ras in an electroporation dish and electroporated with a single current surge of 280 V, 980  $\mu$ F and 1000 ms (Progenetor II, Hoefer Instruments). The cells were diluted with 1% IL-3 in 10 ml of culture medium and incubated at 37°C, 5% CO<sub>2</sub>.

Two days after transfection the cells were diluted 3 times with fresh medium and 250  $\mu$ g/ml of G-418 were added to select for neomycin resistance. On the following days the G-418 concentration was increased to 500, 750 and 1000  $\mu$ g/ml. Dead cells were removed by centrifugation using a Ficoll gradient. For this the cells were first concentrated by centrifugation (5 min, 1200 rpm) in a small volume and layered over Ficoll-Paque (Pharmacia) in

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a centrifuge test tube. After centrifugation (15 min, 2500 rpm, 20°C) and free running from the centrifuge the live cells which had collected on the medium/Ficoll intermediate phase were pipetted off, washed once with medium and transferred into fresh medium.

Eight days after the start of G-418 selection, the IL-3 independent cells began to be concentrated by reducing the IL-3 concentration step by step. After the cells had been cultivated for one week in 0.0005% IL-3, they were separated by seeding in semiliquid Methocel medium. Cell colonies which formed and were well separated from other cells were isolated, transferred into 96-well plates with 100 µl medium (without IL-3) and subsequently expanded. One of the resulting IL-3-independent stable cell lines which exhibited good growth properties was named FHRV12cc21.

### iii) Characterisation of the cells

20 Detection of H-ras<sup>Val12</sup> expression  
FHRV12cc21 and untransfected FDC-P1 cells were grown in IMDM medium with 10% FCS and 1% IL-3. In order to detect the inhibition of the posttranslational modification of H-ras<sup>Val12</sup> by farnesylation the cells were incubated for 48 hours with 5 µM of the protein-farnesyltransferase-inhibitor L-739,749 (Kohl et al., 1994). The cells were washed with PBS and lysed in PBSTDS (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate in PBS) 5 min at 4°C. The cell lysates were clarified by centrifugation (20 min 14,000x g, 4°C). Ras proteins were immunoprecipitated by the addition of 1 µg monoclonal rat antibodies Y13-238 (Oncogene Science) and Protein-G Plus / Protein-A Agarose (Oncogene Science) overnight at 4°C. The precipitates were washed twice with PBSTDS buffer and then extracted in SDS-sample buffer with 3% β-mercaptoethanol. The proteins were separated in a 20% SDS-polyacrylamide

gel, transferred onto an Immobilon membrane (Millipore) and after saturation of the membrane with 10% FCS, 1% bovine serum albumin, 1% Tween-20 in PBS, shown up by successive incubation with a Ras<sup>Val12</sup> specific monoclonal antibody (pan-Ras<sup>Val12</sup>, Oncogene Science) and a secondary anti-mouse-IgG - alkaline phosphatase conjugate by colour reaction with NBT and BCIP (Promega). A clear overexpression of H-Ras<sup>Val12</sup> in FHRV12cc21 cells was demonstrated, compared with the untransfected FDC-P1 control cell and inhibition of the Ras farnesylation by L-739.749. The cells treated with L-739.749 contained more than 50% of the Ras protein in the unmodified (non - farnesylated) form, which therefore migrates more slowly in the SDS gel. (The detection of the overexpression of H-ras<sup>Val12</sup> in FHRV12cc21 cells and the inhibition of Ras-farnesylation by L-739.749 are shown in Fig. 3. After treatment with (+) or without (-) 5  $\mu$ M L-739.749 for 48 h in the presence of 1% IL-3, Ras protein was immunoprecipitated from cell lysates with anti-Ras antibodies and after electrophoretic separation in an SDS-polyacrylamide gel and membrane transfer, detected with anti-Ras<sup>Val12</sup> antibodies. The name "ras<sup>Val12</sup> Std." denotes a standard K-Ras<sup>Val12</sup> preparation (Oncogene Science)).

After treatment with (+) or without (-) L-739.749 for 48 h in the presence of 1% IL-3 Ras protein was immunoprecipitated from cell lysates with anti-Ras-antibodies and after electrophoretic separation in an SDS-polyacrylamide gel and membrane transfer, detected with anti-Ras<sup>Val12</sup> antibodies. The name ras<sup>Val12</sup> Std. denotes a standard K-Ras<sup>Val12</sup> preparation (Oncogene Science).

iv) Inhibition of the IL-3 independent growth of H-ras<sup>Val12</sup> transfected cells by inhibiting Ras farnesylation

40,000 cells were seeded in 100  $\mu$ l RPMI-1640 medium without phenol red (BioWhittaker), supplemented with 10% FCS, 2% glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol, in 96-well plates. Untransfected FDC-P1 cells were cultivated in the same medium with the addition of 0.1% IL-3 containing cell supernatant so that the cells were not forced into apoptosis. A basic solution of the farnesyltransferase-inhibitor L-739.749 (10 mM in DMSO) was serially diluted with PBS with the addition of DMSO and 10  $\mu$ l of the dilutions were pipetted into the cells and then incubated at 37°C and 5% CO<sub>2</sub>. The final DMSO-concentration was 0.5% (v/v) in every case. The number of living cells was indirectly determined by photometric MTS assay (see above). For measuring the DNA synthesis rate, 10  $\mu$ l of [6-<sup>3</sup>H]thymidine in PBS (0.1  $\mu$ Ci; 27 Ci/mmol, Amersham) were added to each well 16 h before the measurement. The cells were transferred onto filter plates using a cell harvester (Canberra Packard), washed with water and the quantity of [<sup>3</sup>H]thymidine incorporated in the DNA was determined in a scintillation counter (TopCount, Canberra Packard). The values shown in Fig. 1 and Fig. 2 correspond to the average from triplicate measurements, based on the untreated cells. (Figs. 1 and 2 show the inhibition of H-ras<sup>Val12</sup> dependent proliferation (viability, given as relative absorption, in Fig. 1; DNA synthesis in Fig. 2) by farnesyltransferase inhibitor L-739.749 in IL-3-free medium. FDC-P1: IL-3-dependent untransfected starting cells.

FDC-P1/J2: IL-3 and Ras-independent FDC-P1 cell line, transformed with activated v-myc plus v-raf. FHRV12cc21: IL-3 independent FDC-P1 cell line, transformed with human H-ras<sup>Val12</sup>.)

b) Preparation of KDR test cells

i) Cloning of KDR and preparation of expression plasmids  
(receptor-DNA)

The cDNA of the human KDR was obtained by screening a  
cDNA bank and cloned into the expression vectors  
5 pRc/CMV (Invitrogen V750-20) or LXSN (Miller, et al.,  
1989).

ii) Isolation of clones which contain the sequence  
coding for human KDR

By screening a human endothelial cell cDNA bank in the  
10 lambda gt11 vector (Clontech HL 1024b) with PCR-probes  
obtained by "nested" amplification of two KDR gene  
fragments (Terman et al., 1992; EMBL Accession Nr.  
X61656) using the DNA extracted from the same bank as  
the starting material, 13 clones were isolated and the  
15 inserts contained in the plasmid pUC18 were sequenced.  
The sense primers used for the PCR-amplification  
corresponded to the amino acid sequences RGQRDLWLWP,  
WDSKKGFTIP , GARFRQGKDYVG, and CWHGEPSQRP, positions  
52-62, 177-186, 940-951 or 1140-1149. The antisense  
20 primers corresponded to the amino acid sequences  
TLVIQAANVSA and SGYHSDDTDTT, positions 514-524 or 1305-  
1315. The full-length cDNA-clone was assembled by  
ligating three overlapping clones and two pairs of  
complementary oligonucleotides, coding for the 38 amino  
25 acids of the published sequence, which does not go up  
to the start-ATG, plus four additional amino acids,  
including the necessary ATG. Nucleotides which promote  
efficient translation of the protein were inserted  
upstream of the ATG (GGATCCCTCGACGCGCC; the BamHI site  
30 at the beginning of this sequence is used for cloning  
purposes). It was possible to ligate the synthetic  
oligonucleotides by creating an EcoRV site at amino  
acids 37-38 (GAC ATA was mutated into GAT ATC, without  
changing the sequence for the amino acids DI). The

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sequences of the cDNA-clones obtained differ from the published sequence (Terman et al., 1992; EMBL-Datenbank, Accession Nr. X61656) at positions 770 (Ala instead of Thr), 785 (Arg instead of Gly), 846 (Val instead of Glu), and 1345 (Ser instead of Thr). These changes make the sequence obtained at the corresponding positions identical to mouse homologues of KDR, Flk-1 (Matthews et al. 1991; GeneBank Accession No. X59397; Millauer et al., 1993; EMBL Accession No. X70842), except at the last position, which is situated in a poorly conserved region. In addition, two of the amino acids in question, namely 770 and 785, are located in the region which was reported to be the transmembranal domain (amino acids 763 bis 787, Terman et al., 1992). It was therefore necessary to make the edge regions similar to groups 763 to 784, corresponding to Flk-1 (Matthews et al., 1991).

iii) Subcloning KDR-cDNA into the expression vector pRc/CMV

The fully assembled KDR-cDNA was first excised from pUC18-KDR with Acc65 (isoschizomer KpnI) and SalI and subcloned into the Acc65 and SalI site of pAD-CMV2 (EP-A 393 438). Then the resulting plasmid pAD-CMV2-KDR was cut with XhoI, the DNA ends were filled using the Klenow fragment of DNA-polymerase I in the presence of all four dNTPs, extracted with phenol and precipitated with ethanol. The blunt end DNA obtained was digested with XbaI. The expression vector pRc/CMV was cut with HindIII, the DNA ends were made blunt, and the vector was then cut with XbaI. The KDR-sequence having a blunt end and an XbaI end which was excised from the plasmid pAD-CMV2 was ligated into this vector, and the resulting plasmid named pRc/CMV-KDR.

- iv) Cloning the KDR-sequence into the expression vector pLXSN

The expression vector pLXSN was cut with Hpa I. The KDR sequence doubly excised from the plasmid pAD-CMV2-KDR with XhoI and XbaI and then blunted by the addition of all four dNTPs using Klenow enzyme was ligated into this vector. A resultant clone of the correct orientation was designated pLXSN-KDR.

10

- v) Transfection of FDC-P1 cells

1 x 10<sup>7</sup> FDC-P1 cells were centrifuged off at 1200 rpm at ambient temperature, washed once with serum-free RPMI and resuspended in 0.8 ml serum-free RPMI. 20 µg pRc/CMV-KDR, linearised with ScaI, were added and electroporated at 280 V, 980 microfarad, 1 sec. Electroporated cells were resuspended in Iscoves Modified Dulbecco's medium (IMDM) with interleukin-3 (IL-3) and 10% foetal calf serum (FCS) and cultivated. Two days after transfection the selection of transfectants was started by the addition of 400 µg/ml geneticin (G418). Another two days later the G418 concentration was increased to 800 µg/ml and subsequently to 1000 µg/ml. Cells killed off by G418 were separated from resistant cells by centrifugation in a Ficoll gradient. To do this, the mixture of dead and living cells was centrifuged, as described above, resuspended in 1 ml IMDM + IL-3 + 10% FCS + 1 g/l G418 and placed over 1 ml Ficoll (Pharmacia). The gradient was run for 15 min at ambient temperature at a speed of 2500 rpm. The living cells are found in the intermediate phase, whilst dead cells are pelleted. The cells of the intermediate phase are washed twice with 10 ml IMDM + IL-3 + 10% FCS + 1 g/l G418 and further cultivated in the same medium.



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In order to obtain G418 resistant cells which grow independently of IL-3, but dependent on VEGF, the IL-3 concentration was reduced to a tenth and at the same  
5 time 10 ng/ml of human recombinant VEGF 165 (Fiebich et al., 1993) was added. Dead cells were again separated from the living ones using Ficoll gradients. The IL-3 was reduced to a tenth of the previous IL-3 concentration, until the cells could be kept  
10 independently of IL-3.

In order to obtain individual clones, these cells were cloned out in Methocel (Metcalf et al., 1992; Meckling-Gill et al., 1992). The cloning out was carried out in  
15 the absence of G418, but in the presence of VEGF.

The cell clones (FDCP-CMV-KDR) were tested for their ability to be stimulated by VEGF. For this, the cells were seeded in 96-well plates (15000 cells/ well) and  
20 cultivated with or without VEGF. After 48 hours an MTS assay (Promega Non radioactive cell proliferation assay: Cell Titer 96AQ) was carried out and the cell growth was determined using this colorimetric assay. None of the clones displayed any increased growth in  
25 the presence of VEGF. As was demonstrated by RT-PCR (Reverse-transcriptase polymerase chain reaction), FDC-P1 cells and the transfected cells (FDCP-CMV-KDR) themselves produce VEGF. FDCP-CMV-KDR cells are thus stimulated autocrinally.

30

vi) Infection of FDC-P1 cells

GP+E-86, a packaging cell line for retroviruses (Markowitz et al., 1988.), was grown in IMDM with  
35 10% FCS in 60 mm culture dishes until 70-80% confluent. These cells were transfected overnight using

lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions with 5  $\mu$ g of uncut (supercoiled) DNA (pLXSN-KDR) and 10  $\mu$ l of lipofectamine. The next day, the cells were detached with trypsin, centrifuged off, taken up in medium, transferred into culture flasks (75 cm<sup>2</sup>) and the selection of transfectants was started by the addition of 800  $\mu$ g G418/ml. After another two days the G418 concentration was increased to 1000  $\mu$ g/ml. After the selection the GP+E-86-LXSN-KDR cells were kept with 10 ng/ml VEGF. To infect FDC-P1 cells, the resistant GP+E-86-LXSN-KDR cells were pooled and 500,000 cells were seeded in IMDM with 10% FCS without G418 in 60 mm culture dishes. 100,000 - 200,000 FDC-P1 cells were added and co-cultivated for 48 hours in the presence of 1% IL-3. The infected FDC-P1 cells (FDCP-LXSN-KDR) were transferred into culture flasks and kept in the presence of VEGF and 1% IL-3. Two days after co-culture, the selection in G418 was started. The selection, IL-3 reduction and cloning out were carried out as described in v) for the transfection of cells (FDCP-CMV-KDR).

c) Preparation of EGF-R (Epidermal Growth Factor Receptor) test cells

EGF-R-test cells designated F/LHERc cells were prepared from the interleukin-3-dependent haematopoietic mouse cell line FDC-P1 by infection with a recombinant retrovirus containing the DNA coding for the human EGF-R. The recombinant retrovirus used for the expression of human EGF-R-cDNA (Ullrich et al., 1984) corresponds to the construct described by von Rüden, T. et al., 1988, except that the retroviral vector LXSN (Miller al., 1989) was used for the expression of EGF-R cDNA (as in a) for using

KDR) and the packaging cell used was the line GP+E86 (Markowitz al. 1988). Selection and cloning of the cells were carried out essentially as described in a). In order to select virus-infected FDC-P1 cells the cultures were  
5 selected out in 1000  $\mu$ g/ml G418 in the presence of 1% IL-3 (1% conditioned medium of the IL-3-secreting cell line X63-0 mIL-3; Karasuyama and Melchers, 1988), in order to ensure maximum IL-3 stimulation, as the virus construct also carries a neomycin resistance gene next to the human  
10 EGF-R cDNA. Then 20ng/ml human recombinant EGF (Promega) was added to the cultures of G418-resistant cells and the IL-3 concentration was successively brought back from 1% to zero. Cells which grew EGF-dependently in the absence of IL-3 were finally cloned in methylcellulose in the  
15 presence of TGF- $\alpha$ , but not IL-3 (like EGF, TGF- $\alpha$  also stimulates the EGF receptor, but is more stable). Finally it was confirmed, with each isolated clone, that the cells die in the absence of EGF and IL-3, that they proliferate in the presence of EGF or IL-3 as the sole  
20 growth factor, and that the EGF stimulation is cancelled out by known EGF receptor inhibitors.

d) Preparation of HER2 test cells

- i) Subcloning of the mutant HER2 sequence into the  
25 expression vector pAHygCMV1

From plasmid pHer2-CVN, which contains the complete 4.5 kb coding sequence of the human receptor HER2 (GeneBank AC-No. M11730; Coussens et al., 1985), the HER2-cDNA was excised in 2 parts and subcloned: First the 3.2 kb SalI-  
30 KpnI fragment which contains the 5'-part of the HER2-cDNA was excised and cloned in a directed manner into the expression vector pAHygCMV1 doubly cut with SalI and KpnI, so that the transcription of HER2 is under the control of the cytomegalovirus (CMV) promoter/enhancer

element. The resulting intermediate product was cut first with NotI, the ends were made blunt ("blunt ended") by the addition of dNTPs and the Klenow fragment of DNA-polymerase, and then cut with KpnI. Then the 0.8 kb KpnI-StuI fragment which contains the 3'-part of the HER2-cDNA was excised from pHer2-CVN and directedly cloned into the intermediate construct. The expression vector pAHygCMVHer2 formed contains the complete coding region of HER2 under the control of the CMV promoter.

10 In order to introduce the point mutation Val659->Glu659 (GTT->GAA) (Suda et al., 1990) the 3.2 kb SalI-KpnI HER2-fragment was first cloned into the plasmid pBluescript KS (Stratagene), which had previously been opened with SalI and KpnI. For the mutagenesis, an oligonucleotide which

15 contains the AatII cutting site and, 16 bp downstream, the mutated sequence GAA, and an antisense oligonucleotide which contains the sequences around the NdeI cutting site, were synthesised. Using these two oligonucleotides as primers the 400 bp AatII-NdeI

20 fragment was amplified by PCR (polymerase chain reaction) from the SalI-KpnI HER2 fragment which now contains the mutation GTT->GAA. The amplified fragment was recut with AatII and NdeI and cloned into the plasmid pBluescript/Her2(SalI-KpnI) which had also been cut with

25 AatII and NdeI. The region from AatII to NdeI was sequenced to ensure that no mutations other than those desired were obtained as a result of the PCR amplification. Plasmid pBluescript/Her2(V659E) now contains the mutated form of the 3.2 kb SalI-KpnI HER2-

30 fragment. This mutated fragment was re-excised from the plasmid with SalI and KpnI, the non-mutated fragment was also excised from the expression vector pAHygCMVHer2 with SalI and KpnI, and replaced by the mutated fragment. The resulting expression vector, known as

35 pAHygCMVHer2(V659E), now contains the complete coding

region of the mutated, constitutively active form  
HER2 (V659E) .

ii) Transfection of the FDCP cells

5

FDC-P1 cells (Dexter et al., 1980), which grow IL-3-  
dependently, were transfected with plasmid  
pAHygCMVHer2(V659E) as follows: About  $1 \times 10^7$  cells were  
mixed with 10  $\mu$ g plasmid DNA in IMDM-medium (Bio-  
10 Whittaker) and electroporated with an Electroporator  
(Hoefer, Progenetor II) at 980  $\mu$ F, 280 V for 1000 ms. The  
cells were kept in IMDM-medium in the presence of 10%  
heat-inactivated foetal calf serum, 50  $\mu$ M  $\beta$ -  
mercaptoethanol, standard antibiotics, and 1% IL-3 at  
15 37°C under 5% CO<sub>2</sub> and selected by the addition of 300  
mg/l hygromycin B (Boehringer Mannheim). After successful  
selection the cells were slowly weaned off IL-3, then the  
IL-3-independently growing cell pool was subcloned in  
Methocel. Individual clones were picked and cultivated in  
20 medium without IL-3.

iii) Testing the usefulness of the cells

Individual clones were tested in a fluorescence-activated  
25 cell sorter (Becton-Dickinson, FACSort) for surface  
expression of HER2, using as the primary antibody a mouse  
antibody directed against the extracellular domain of  
HER2 (Oncogene Science, c-neu (Ab-2)), and as the  
secondary antibody a fluorescein isothiocyanate (FITC)-  
30 conjugated anti-mouse IgG antibody (Dianova, Hamburg).  
The subclone with the best surface expression of  
HER2 (V659E), known as FDCP/Her2-C42#16, was used for the  
screening.

e) Preparation of HGF (Hepatocyte Growth Factor) receptor test cells

i) Subcloning of the c-met sequence into the expression vector pLXSN

5

The complete 4.6 kb coding sequence of the human HGF receptor c-met (GeneBank AC-No. J02958; Park M., et al., 1987), was directedly cloned into the expression vector pLXSN doubly cut with EcoRI and XhoI. Plasmid pLXSN is a  
10 retroviral vector which is used to produce recombinant retroviruses (Miller, et al. 1989). The expression vector pLXc-met formed contains the complete coding region of c-met.

15 ii) Transfection of the packaging cell line GP+E86

The packaging cell line GP+E86, a mouse fibroblast cell line (Markowitz et al., 1988), was transfected with plasmid pLXc-met as follows: About  $1 \times 10^6$  cells per 10 cm  
20 plate were seeded in DMEM-medium (Bio-Whittaker) in the presence of 10% heat-inactivated foetal calf serum (FCS) in a number of plates. The next day 20  $\mu$ g of plasmid-DNA was precipitated with calcium phosphate and the precipitate was slowly added dropwise to the cells. After  
25 4 hours the cells were incubated for 2 min in 15% glycerol, washed and then kept in DMEM-medium in the presence of 10% heat-inactivated foetal calf serum and standard antibiotics at 37°C under 5% CO<sub>2</sub>. The cells were selected by the addition of 1 g/l G418 (GIBCO BRL). Cell  
30 clones were pooled and the cell pool was cultivated.

The cell supernatant should contain replication-defective recombinant virus particles which infect cells of murine origin. In order to determine the virus titre,  $5 \times 10^4$  NIH-  
35 3T3(tk<sup>-</sup>) cells were seeded per 6 cm dish and incubated

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with various concentrations of the cell supernatants (1 ml supernatant + 8  $\mu$ g/ml Polybren). Cells were selected in G418 and the virus titre was determined from the number of clones.

5

iii) Infection of the FDC-P1 cells

FDC-P1 cells (Dexter et al., 1980) which grow IL-3-dependently were infected with recombinant virus particles as follows: About  $2 \times 10^6$  cells were incubated in 10 2.5 ml virus supernatant of the GP+E86/LXc-met cells and 8  $\mu$ g/ml of Polybren and 1% IL-3 for 4 hours at 37°C. The cells were diluted in IMDM-medium in the presence of 10% heat-inactivated foetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, standard antibiotics and 1% IL-3 and 15 kept at 37°C under 5% CO<sub>2</sub>. The selection was carried out by the addition of 1 g/l G418 (GIBCO BRL). Antibiotic-resistant stable cell lines were then selected for HGF-dependent and IL-3-independent growth by adding HGF and removing IL-3. To do this, the cell pool still growing 20 dependent on IL-3 was first subcloned in Methocel. Clones which were still IL-3-dependent clones were picked out and individually slowly weaned off IL-3 by keeping the clones in 20 ng/ml recombinant HGF (Sigma) as growth factor while the IL-3 concentration was reduced more and 25 more. Individual HGF-dependent but IL-3-independent clones were cultivated.

iv) Testing the usefulness of the cells

In order to establish whether the clones grow HGF-dependently, the cells were seeded in various 30 concentrations of HGF and the growth of the cells was determined by the incorporation of <sup>3</sup>H-thymidine: For this, cells were seeded in microtitre plates (at a concentration of 20000 cells per well) and incubated with 35 various concentrations of recombinant HGF (Research &

Development). After 2 days  $^3\text{H}$ -thymidine was added overnight and the incorporation of thymidine was determined; in addition, as stated in Example 1a), the number of living cells was determined indirectly by a photometric MTS assay using absorption (Fig. 4) measured the next day (Packard, TopCount). Positive clones were additionally tested in a fluorescence-activated cell-sorter (Becton-Dickinson, FACSORT) for surface expression of c-met, using as primary antibody a murine antibody directed against the extracellular domain of c-met (Upstate Biotechnology, Inc., Cat.# 05-237), and as secondary antibody a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Dianova, Hamburg). The subclone with the best surface expression of c-met, known as FDCP/LXc-metH6, was used for the screening.

f) High Throughput Screen

The test cells obtained according to a) to e) were cultivated in RPMI/1640 medium (BioWhittaker), supplemented with 10% foetal calf serum (FCS; Boehringer Mannheim), 2 mM glutamine (BioWhittaker), at 37°C and 5% CO<sub>2</sub>, in the presence of the corresponding growth factors. In order to investigate the inhibitory activity of the compounds being studied (test substances)  $1.5 \times 10^4$  cells were cultivated in 100  $\mu\text{l}$  medium per well in 96-well plates in the abovementioned medium with the addition of the corresponding growth factors. The chosen test substances were present in a concentration of 5 mg per ml dissolved in 100% dimethylsulphoxide (DMSO) and were added to the cultures in a dilution of 1:1000, whilst the DMSO concentration did not exceed 0.1%. The final concentration of the test substances was 5  $\mu\text{g/ml}$  in the test system, each substance was tested in 3 microtitre plate wells (triplicate measurement). On each plate three positions were treated only with DMSO in the corresponding dilution



(1:1000); these positions were used as a negative control. The cultures were incubated for 21 h at 37°C under cell culture conditions. After the end of the incubation period 20 µl of a mixture of MTS (0.88 mg/ml; Promega G511A) and PMS (0.11 mg/ml; Sigma P-9625) were added and incubated for a further 3 hours at 37°C. Once the incubation period had ended, the test plates were measured in a microtitre plate photometer and the data were evaluated in a data bank. The relative cell number which correlates with the extinction at 492 nm was given as a percentage of the negative control of the test plate.

g) Comparative screening and testing for specificity

The High Throughput Screen described in f) was carried out parallel to the test cell lines described in a) to e). The same dilutions of the test substance were simultaneously added to the cells placed in various microtitre plates, which were subsequently kept under exactly the same conditions and evaluated. Substances which, in a test system, had a value of less than 50% of the negative control value on the same plate, were regarded as potential hits and investigated more closely for specificity. A substance was regarded as specific if, in the assay in question, it had a value of less than 50% of the negative control and in the parallel assays achieved a value of more than 75% of the corresponding negative control. These substances were selected and examined in a detailed dosage-activity curve for their IC<sub>50</sub>-value. The cells were placed in a microtitre plate under the same conditions as described in f) and investigated with a serial dilution of the corresponding test substance in concentrations of between 400 µM and 10 nM. The IC<sub>50</sub> value is the concentration of test substance in which the test value reaches 50% of the negative control. Table 1 shows a

summary of the results obtained from tests with a larger quantity of test substances.

A total of 231360 tests yielded 2834 "hits", i.e.

substances which satisfy the criterion of "inhibition" to  
5 less than 50% of the control, corresponding to a

percentage of 1.22% of the substances tested. As a rule, in screening to find active substances, the concentrations of the test substances are adjusted so that about 1-2% of the test substances meet the criteria specified. These

10 substances then have to be investigated manually and with considerable effort for their specificity and mechanism of activity. This is generally done by detailed tests with various cellular and enzymatic assays. In high throughput screening this would represent substantial labour and

15 other costs. The parameters set by the comparative screening according to the present invention narrow down the range of interesting substances needing investigation in detailed secondary assays to about 10% of the original hits. After detailed investigations 13 substances which  
20 selectively block the target molecule in question still remained (4.3% of the hits regarded as specific),

constituting a guiding structure for the development of active substances.

25 The specificity of an inhibitor for the EGF-R is illustrated by the example of 4-[(3-chlorophenyl)-amino]-6-(cyclopropylamino)-pyrimido-[5,4-d]pyrimidine in Fig. 7. Whereas the IC<sub>50</sub> value for the EGF-dependent cell line (in Fig.7: "EGF auto5") is about 500 nM, on the other test  
30 cell lines containing Ras (in Fig.7: "FHR V12"), HER-2 or HGF-R (in Fig.7: "TPRmet") as target molecules, effects on the proliferation of the cells can only be measured at a concentration 100 times greater. The failure rate of 95% after detailed tests can be explained by the fact that the  
35 test cells do indeed have an identical genetic background, but are clonal in origin, on the one hand, and ectopically

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express different target molecules, on the other hand. As a result there are slight differences in sensitivity compared with nonspecifically active organic substances.

## 5 Example 2

Luciferase assay in the High Throughput Format for screening substances with a specific effect on receptor-tyrosinekinase-dependent signal transmission pathways

10

a) Preparation of recombinant KB cells, which express luciferase as a function of HGF or EGF.

i) Subcloning of the c-fos promoter region into the expression vector pBHlucOL

15 The promoter region of the human c-fos gene was excised from plasmid pfosCAT (Schönthal, A. et al., 1988) with XbaI and HindIII and subcloned first into plasmid pBluescript SK (Stratagene). The resulting intermediate product was first cut with XbaI, the ends were made blunt ("blunt ending") by the addition of dNTPs and the Klenow fragment of DNA-polymerase and then cut with SalI. Plasmid pBHlucOL, which contains the luciferase gene (Voraberger, G. et al., 1991), was cut first with HindIII, the ends were made blunt by the addition of  
20 dNTPs and the Klenow fragment of DNA-polymerase, then cut with SalI, and the fos-promoter fragment was cloned into this, resulting in plasmid pBHfosluci.

25

ii) Transfection of the KB cells

30

The human epidermoid carcinoma cell line KB (ATCC CCL 17) was transfected with plasmid pBHfosluci as follows: about  $1 \times 10^6$  cells per 10 cm plate were seeded in DMEM-medium

(Bio-Whittaker) in the presence of 10% heat-inactivated foetal calf serum in several plates. The next day 20  $\mu$ g of plasmid-DNA were precipitated with calcium phosphate and the precipitate was slowly added dropwise to the cells.

- 5 After 4 hours the cells were incubated for 2 min in 15% glycerol, washed and then kept in DMEM medium in the presence of 10% heat-inactivated foetal calf serum and standard antibiotics at 37°C under 5% CO<sub>2</sub>. The cells were selected by the addition of 0.5 g/l G418 (GIBCO BRL).
- 10 Individual G418-resistant cell clones were cultivated and tested for the inducibility of luciferase expression by the addition of HGF (Sigma). Approximately 10000 cells of each clone were seeded 6-fold in 200  $\mu$ l per well in a light-impermeable 96-well microtitre plate covered with
- 15 tissue culture (Microlite™, Dynatech Laboratories) and incubated overnight at 37°C. Three batches were treated with 20 ng/ml HGF and incubated for a further 6 h. Then the medium was removed and the cells were washed twice with PBS. The cells were taken up in 150  $\mu$ l of lysing
- 20 buffer (25 mM Tricin, 0.5 mM EDTA, 0.54 mM sodium tripolyphosphate, 6.5 mM DTT, 16.3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1% Triton X-100, 1.2 mM ATP, 0.05 mM luciferin; pH7.8) per batch and the luciferase activity was measured in a 96-well luminometer (ML-1000, Dynatech). Cell clone KB-B4
- 25 was chosen for further experiments, as it had a measurable base level of luciferase activity as well as a 10-15-fold inducibility by HGF.

iii) Testing the usefulness of the cell line KB-B4

- 30 In order to determine whether the luciferase gene in clone KB-B4 can be stimulated by HGF or EGF the cells were seeded in various concentrations of HGF or EGF and the luciferase activity was measured: For this the cells were seeded in microtitre plates in a concentration of
- 35 10000 cells per well and incubated with various

concentrations of HGF or EGF. After 6 hours the cells were lysed as above and the luciferase activity was measured. A dosage-dependent induction was apparent, with a maximum induction of 10- or 20-fold. An Example is shown in Fig.5.

Since various inhibitors of tyrosinekinase activity of the EGF receptors are known, KB-B4 cells were incubated for 6 hours with various concentrations of different inhibitors with the addition of 1 ng/ml of EGF and then the luciferase activity was measured. All the inhibitors tested produced a dosage-dependent inhibition of luciferase induction by EGF. An example of the inhibitory activity of an inhibitor is shown in Fig.6, taking the example of the test substance 6,7-dimethoxy-4-(3-methylphenylamino)quinazoline.

#### b) High Throughput Screen

The KB4 cells described in a) for testing for EGF and HGF were propagated in DMEM (Bio Whittaker), supplemented with 10% FCS and 500 mg/ml G418. In order to investigate the test substances,  $1 \times 10^4$  cells were seeded in 100  $\mu$ l phenol-red-free DMEM/1% FCS per well in 96 well microtitre plates. Chosen test substances were present in a concentration of 5 mg per ml dissolved in 100% dimethyl sulphoxide (DMSO) and were added to the cultures in a dilution of 1:600, the DMSO concentration not exceeding 0.25%. The final concentration of the test substances was about 8.3  $\mu$ g/ml in the test system, each substance was tested in three microtitre plate wells (triplicate measurement). On each plate three positions were treated only with DMSO in the corresponding dilution (1:600); these positions were used as a negative control. After the addition of the test substances the test cells were induced with EGF (20 ng/ml) or HGF (20 ng/ml) in order to

10

c) Comparative screening<sup>†</sup> and testing for specificity

15

20

25

30

Table 1

Assay	Hits	Hits %	Specific hits	specific hits in %
EGF	28	1.04	7	0.26
HGF	27	1.00	6	0.22

5

### Example 3

Luciferase assay in the High Throughput Format for  
screening substances with a specific effect on signal  
transmission pathways which are dependent on G protein-  
coupled receptors

a) Preparation of three different recombinant A549 test  
cell lines which express luciferase as a function of the  
activation of the human neurokinin-1(NK1) receptor, the  
neurokinin-2(NK2) receptor or the serotonin receptor 5HT<sub>2</sub>

The preparation and cultivation of the NK2 cell line  
(A20/NK2-122) and the 5HT<sub>2</sub> cell line were carried out as  
described in Example 6 of WO 93/11257. In order to prepare  
the NK1 cell line (A20/NK1-12A) the cDNA of the human NK1  
receptor (Gerard et al., 1991) was cloned into the  
expression vector pAHygCMV1 analogously to NK2 and in this  
way the pretest cell line was transfected. The rest of the  
procedure and the cultivation were carried out in the same  
way as for the NK2 test cells.

b) High Throughput Screen

The cell lines prepared in a) for testing for NK1 and NK2 are propagated in RPMI/1640 medium (BioWhittaker),  
5 supplemented with 10% dialysed FCS, 0.8 g/l G418 and 0.15 g/l hygromycin. To investigate the test substances  $1 \times 10^4$  cells are seeded in 100  $\mu$ l of UltraCulture medium (BioWhittaker) per well in 96 well microtitre plates. Chosen test substances were present in a concentration of  
10 5mg/ml dissolved in 100% dimethylsulphoxide (DMSO). They were added to the cultures in two concentrations of 1:300 and 1:3000, with the DMSO concentration not exceeding 0.5%. The final concentration of the test substances was about 16.6 and 1.66  $\mu$ g/ml in the test system, with each  
15 substance being tested in four microtitre plate wells per dilution. On each plate four positions per dilution were treated only with DMSO in the corresponding dilution (1:300, 1:3000). These positions were used as a negative control. After the addition of the test substances, the  
20 NK1 cells were mixed with substance P (1  $\mu$ M) to induce the TRE-sensor-DNA, the NK2 cells were mixed with neurokinin A (10  $\mu$ M) and the 5HT<sub>2</sub> cells with serotonin (100  $\mu$ M). After an incubation period of 7 h at 37°C and 5% CO<sub>2</sub> the microtitre plates were washed 4 times with 150  $\mu$ l of  
25 washing buffer per well (25 mM Tricin, 16.3 mM MgSO<sub>4</sub>). Then 100  $\mu$ l lysing buffer (25 mM Tricin, 0.5 mM EDTA, 0.54 mM sodium tripolyphosphate, 16.3 mM MgSO<sub>4</sub>, 1.2 mM ATP, 0.05 mM luciferin, 56.8 mM  $\beta$ -mercaptoethanol, 0.1% Triton X-100, pH 7.8) were added and the luminescence  
30 was measured in a Dynatech Luminometer and the data were evaluated in a data bank. The relative promoter activity which correlates with the luminescence measured was given as a percentage of the negative control of the test plate.

35 c) Comparative screening and testing for specificity



The High Throughput Screen described in b) was carried out parallel to the three cell lines mentioned above. The same dilutions of the test substances were simultaneously added to the cells present in various microtitre plates, which  
5 were subsequently kept under exactly the same conditions and evaluated. Substances whose results were less than 40% of the negative control in one test system and were more than 65% of the negative control in the other parallel assays were regarded as specific inhibitors of the test  
10 systems in question. Such substances were tested a second time in a corresponding High Throughput Screen to check their reproducibility. Substances whose results were repeatable were selected and subjected to additional tests. Some substances also inhibit two of the three test  
15 systems, some nonspecifically inhibit all three assays carried out in parallel. Table 2 shows a summary of the activity of 1458 substances on the tests described.

Table 2

20

Assay	Number of hits	in %
only NK1, not NK2, 5HT2	5	0.34
only NK2, not NK1, 5HT2	18	1.23
NK1 and NK2, not 5HT2	6	0.41
only 5HT2, not NK1, NK2	74	5.07
NK1, NK2 and 5HT2	61	4.18

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555T00-13630260

Patent Claims

1. Process for determining the pharmacological effect of  
5 a substance on the activity of various biological  
target molecules, wherein a substance is applied to  
test cells which contain one or more biological  
target molecules and the effect of the substance on  
10 the activity of the target molecules is determined,  
characterised in that in one operation a defined  
amount of a test substance
- a) is applied to test cells with the same basic  
biological constitution which differ in that they  
15 contain one or more different biological target  
molecules; and/or
- b) is applied to test cells which contain one or  
more biological target molecules, the cells differing  
20 in that they have different basic biological  
constitutions, and
- i) the effect of the substance on the or each  
biological target molecule is measured using a  
25 detection system coupled to the activation of the  
target molecule; and/or
- ii) the effect of the substance on different  
regulatory mechanisms triggered by the activation of  
30 the target molecule is determined by measuring the  
effect using a plurality of detection systems each  
coupled to the different regulatory mechanisms,  
and the effects of the test substance on the  
different test cells or the effects determined using  
35 different detection methods are directly compared  
with one another.

2. Process according to claim 1, characterised in that a plurality of substances, optionally in several dilutions, are applied in parallel to one or more sets of cellular substrates, each set constituting a group of different assays or assay formats based on the same starting cell.
3. Process according to claim 1 or 2, characterised in that the test cells are mammalian cells.
4. Process according to claim 3, characterised in that the test cells are human cells.
5. Process according to one of the preceding claims, characterised in that the test cells endogenously express the target molecule in question.
6. Process according to one of claims 1 to 4, characterised in that the test cells are transformed with the DNA coding for the target molecule in question.
7. Process according to one of the preceding claims, characterised in that the substance is also applied to control cells which do not contain the target molecule in question and the effect of the test substance on the test cells is compared with their effect on the control cells.
8. Process according to one of the preceding claims, characterised in that the target molecule is a receptor.
9. Process according to claim 8, characterised in that the receptor is a tyrosinekinase.

10. Process according to claim 9, characterised in that the receptor is the EGF receptor.
- 5 11. Process according to claim 9, characterised in that the receptor is HER2.
12. Process according to claim 9, characterised in that the receptor is the HGF receptor.
- 10 13. Process according to claim 9, characterised in that the receptor is KDR.
14. Process according to claim 8, characterised in that the receptor is a G protein-coupled receptor.
- 15 15. Process according to claim 14, characterised in that the receptor is a neurokinin receptor.
- 20 16. Process according to claim 14, characterised in that the receptor is a serotonin receptor.
17. Process according to one of claims 1 to 7, characterised in that the target molecule in question is an intracellular component of a signal transmission pathway.
- 25 18. Process according to claim 17, characterised in that the target molecule is a protein kinase.
- 30 19. Process according to claim 17, characterised in that the target molecule is ras.
20. Process according to claim 17, characterised in that the target molecule is raf.
- 35

21. Process according to one of claims 1 to 7,  
characterised in that the target molecule in question  
is a molecule which participates in apoptosis.
- 5 22. Process according to claim 21, characterised in that  
the target molecule is bcl-2.
- 10 23. Process according to claim 1, characterised in that  
the test cells contain a reporter gene under the  
control of a regulatory sequence which responds to  
the change in the concentration of a messenger  
substance of a signal transmission pathway, of which  
the target molecule in question is a component, and  
that the effect of the test substance on the target  
15 molecule is determined in a change in the expression  
of the reporter gene.
- 20 24. Process according to claim 23, characterised in that  
the reporter gene is luciferase.
- 25 25. Process according to claim 23, characterised in that  
the reporter gene is Green Fluorescent Protein.
- 30 26. Process according to claim 1, characterised in that  
test cells which are dependent on a growth factor for  
their proliferation are cultivated in the presence of  
the growth factor and the effect of the substance on  
the cells is determined by directly or indirectly  
measuring the apoptosis or the proliferation of the  
cells.
- 35 27. Process according to one of the preceding claims,  
characterised in that it is carried out in the High  
Throughput Format.



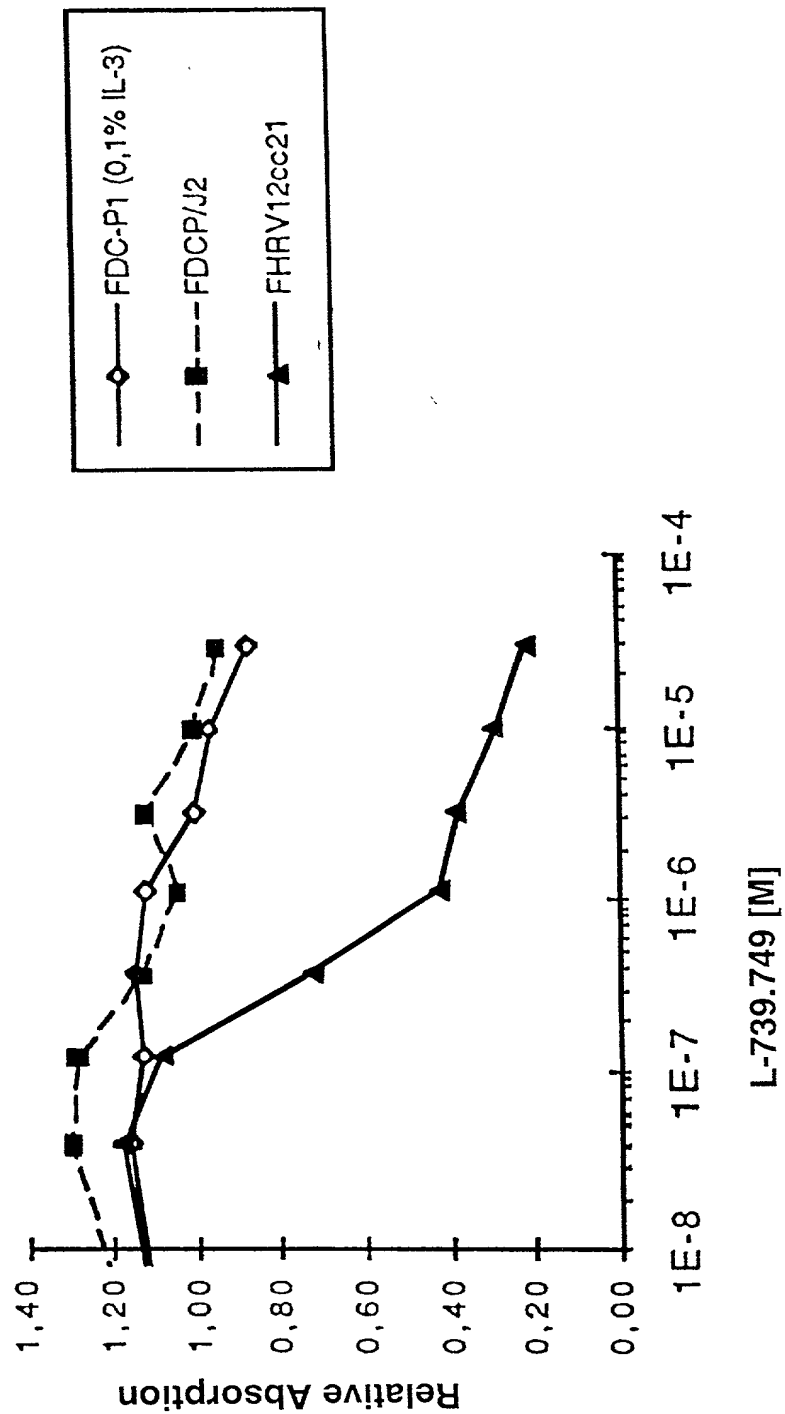
# Abstract

5 Process for the comparative high throughput screening of  
substances with a pharmacological effect. The substances  
are applied, in one operation, to test cells which contain  
one or more biological target molecules, the cells having  
an identical basic biological constitution and differing  
10 in the target molecule, and/or to cells with different  
basic biological constitutions and identical target  
molecules. The effect of the substance on the activity of  
the target molecules is measured using a detection system  
coupled to the activation of the target molecule and  
15 compared directly with the effect on other target  
molecules.

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FIG. 1.

MTS: 40,000 cells, 24h



Thymidine Incorporation: 40,000 cells, 24h

FIG. 2.

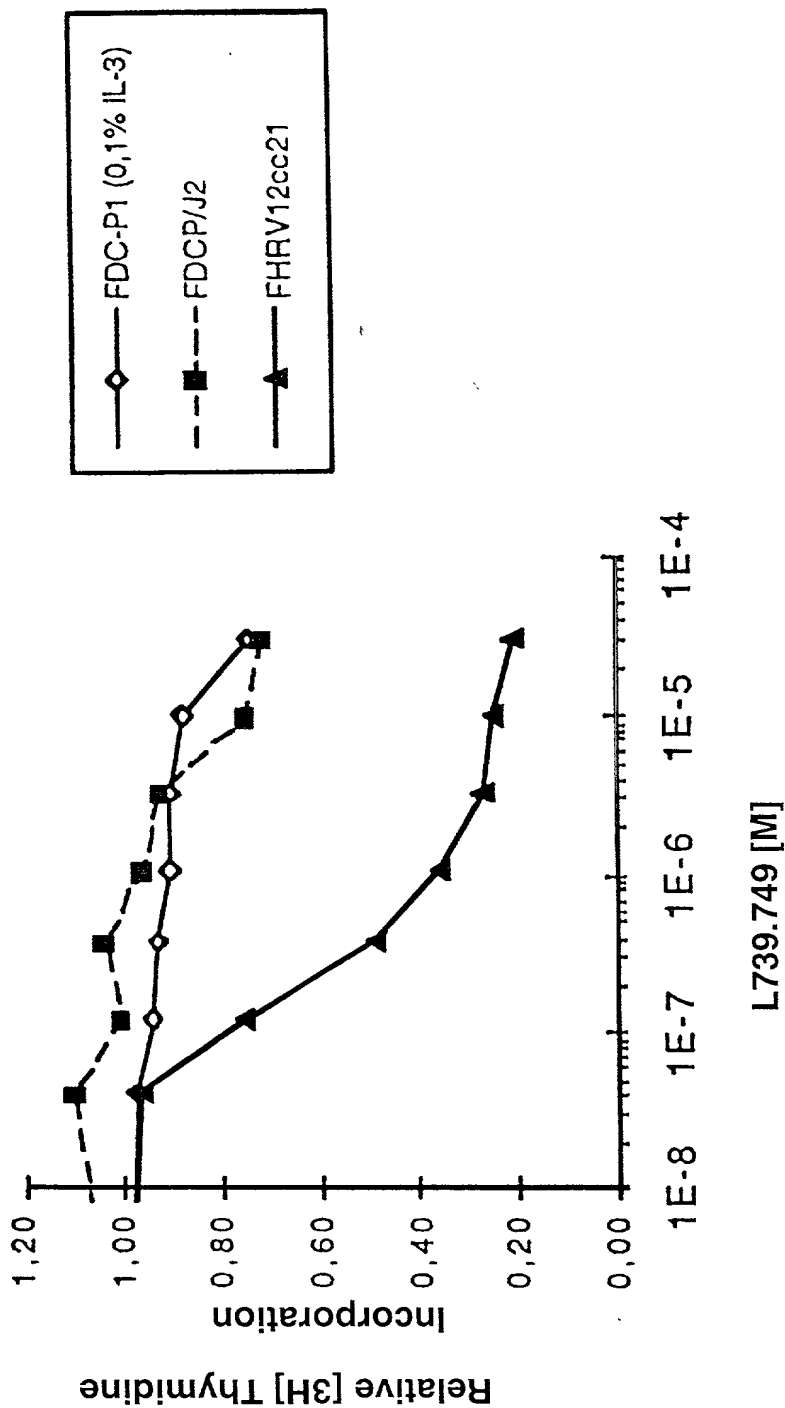


FIG. 3.

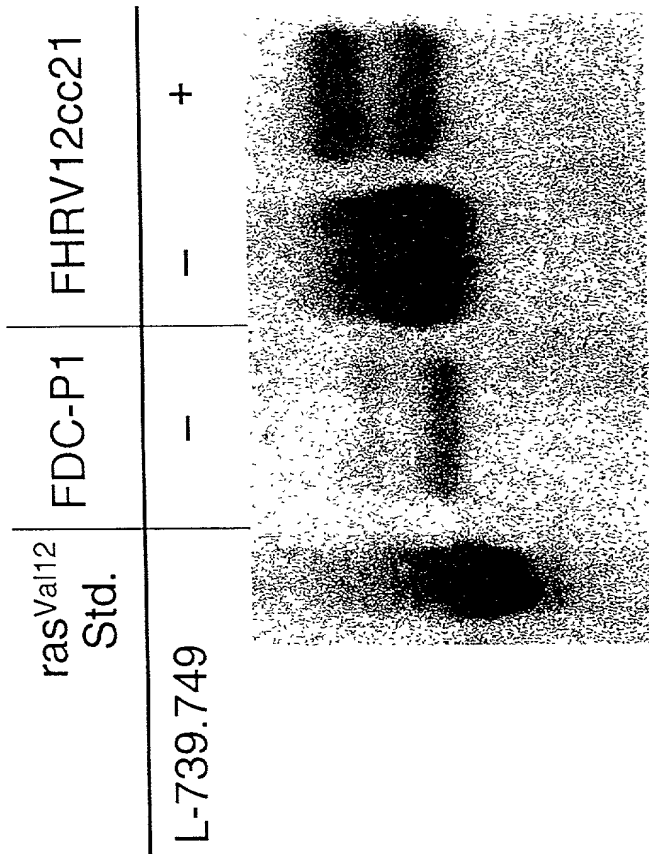


FIG. 4.

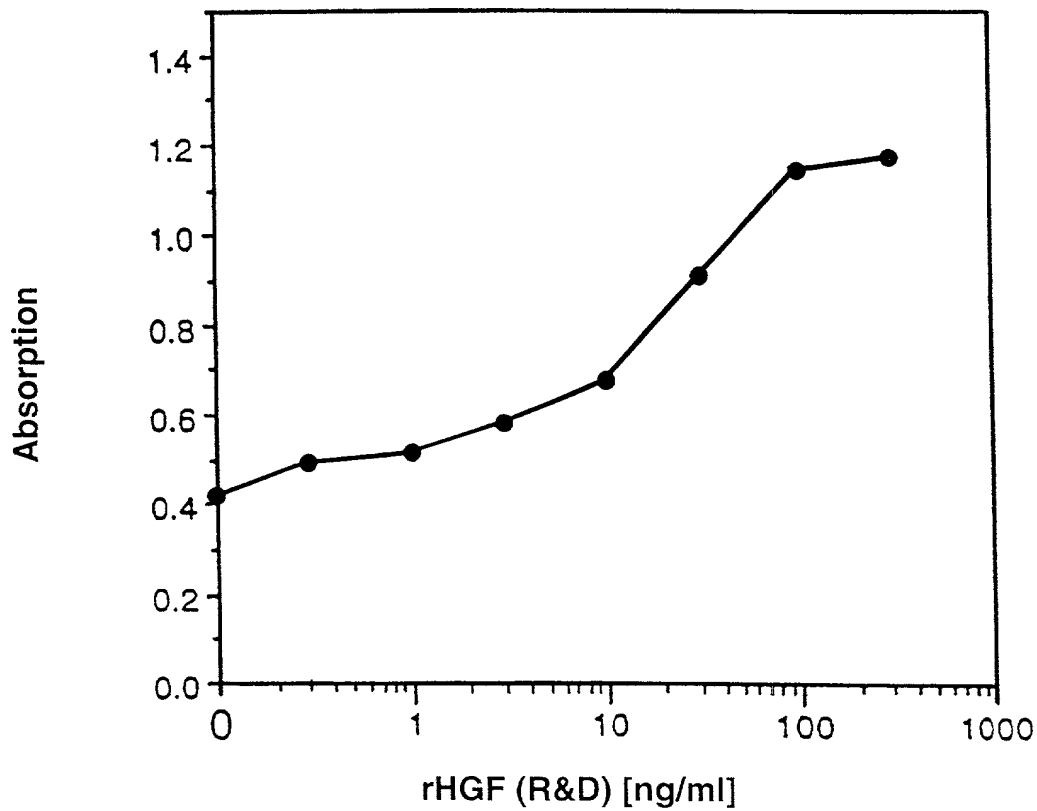
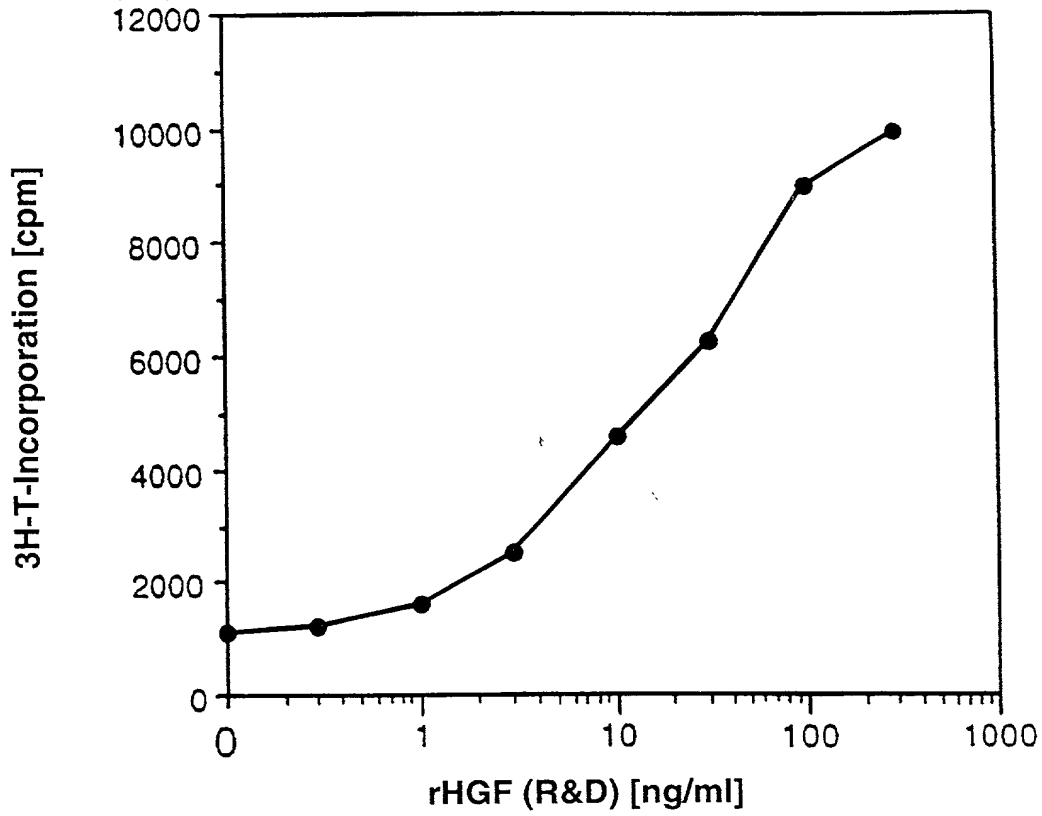
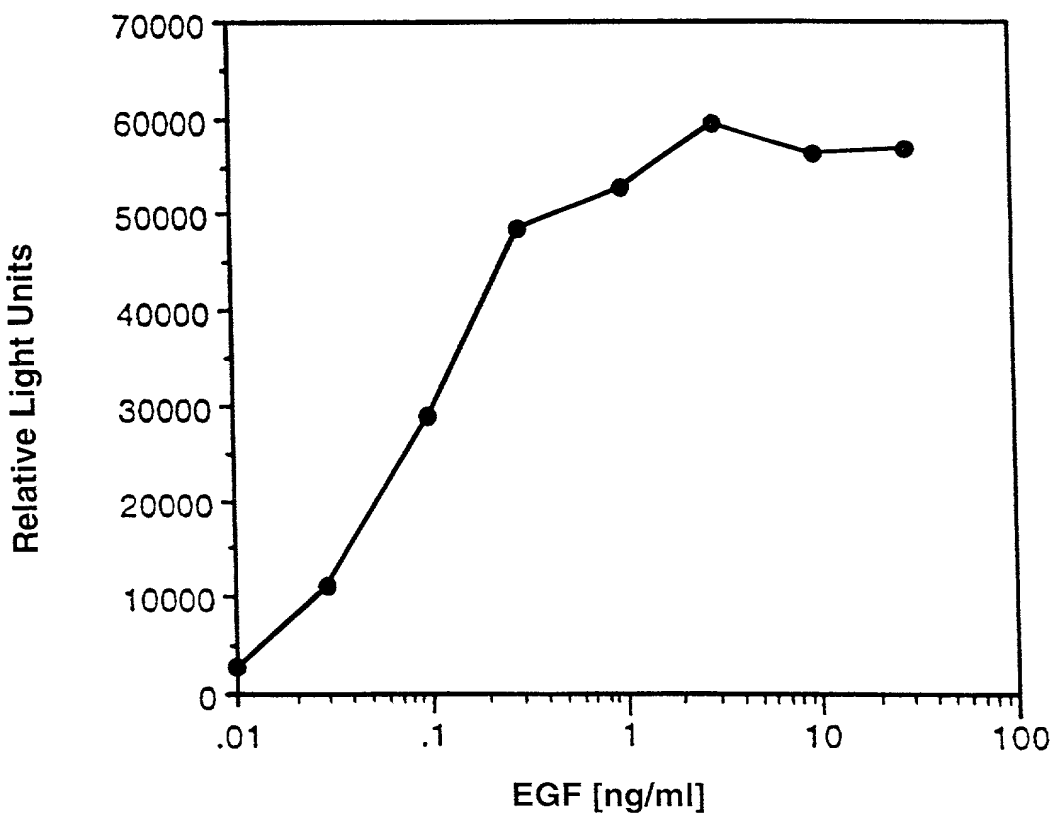
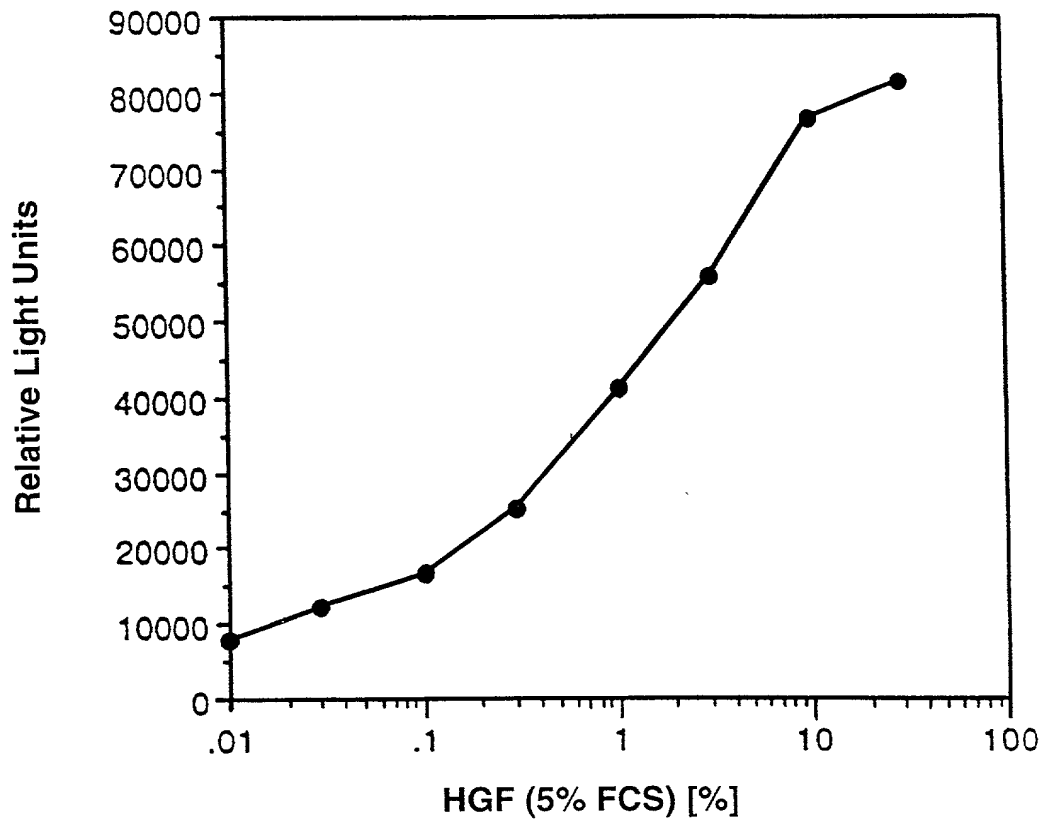


FIG. 5.



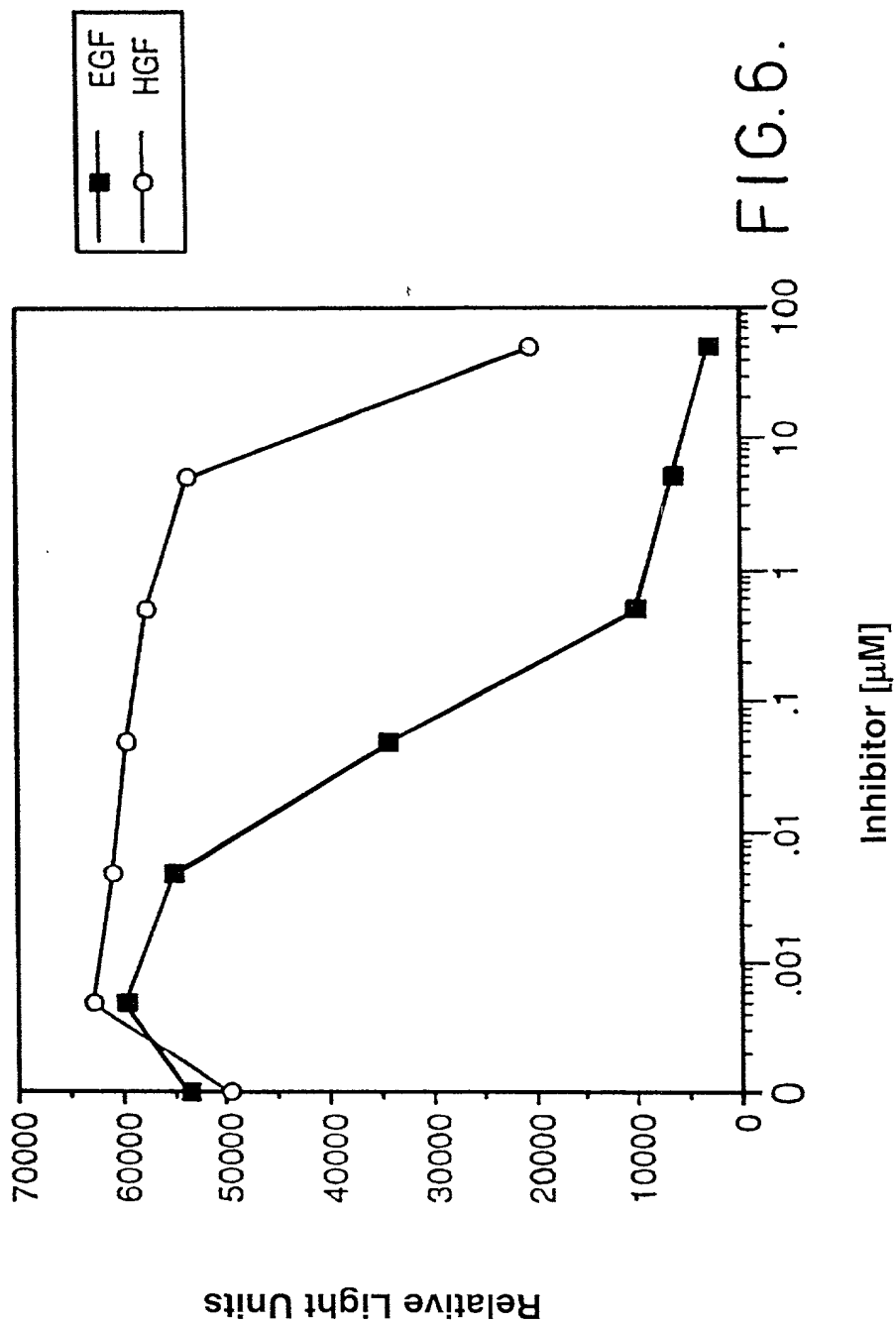
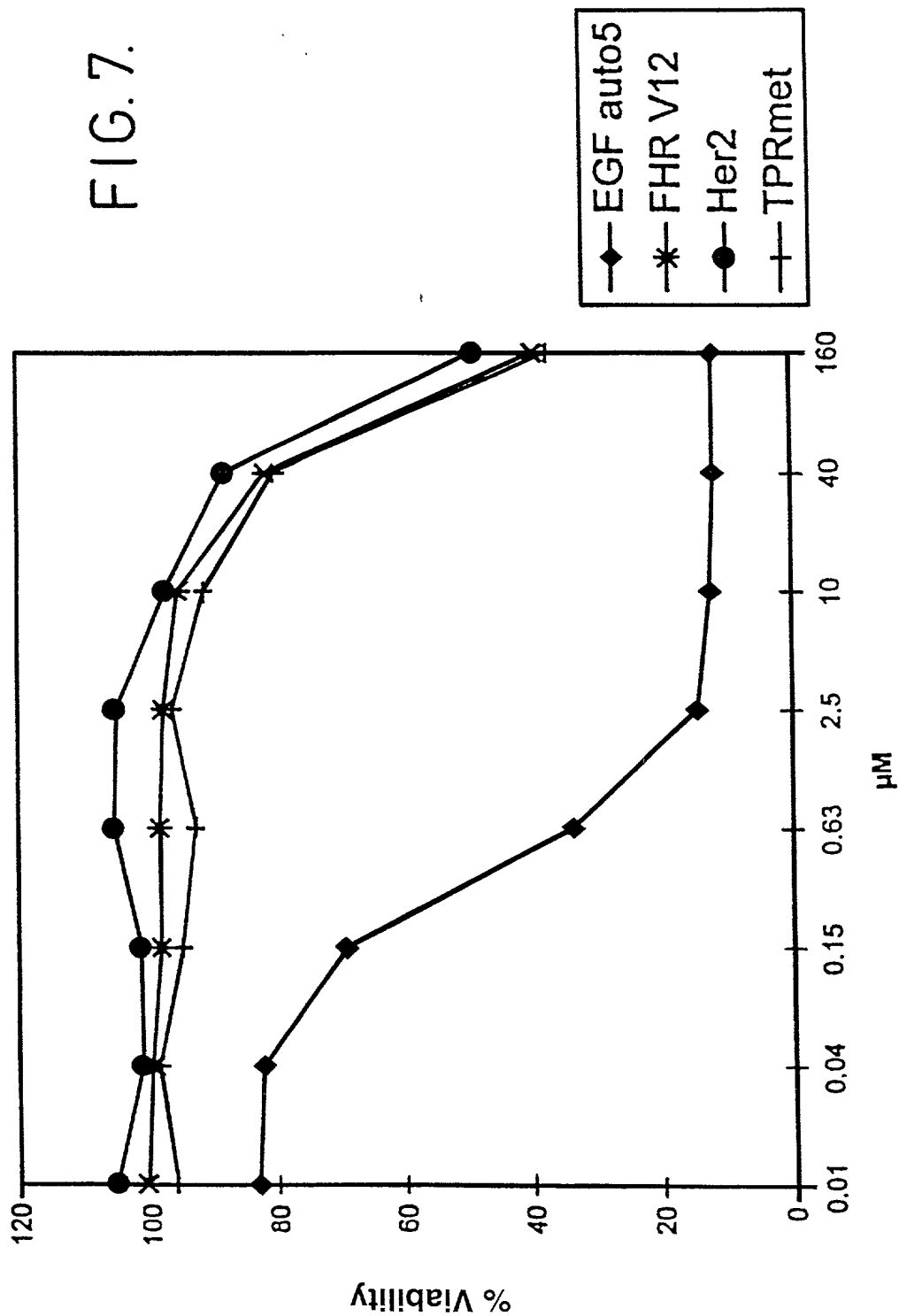


FIG. 7.





## POWER OF ATTORNEY FROM ASSIGNEE

09/202984

Boehringer Ingelheim International GmbH, a corporation of Germany, having a principal place of business at Binger Straße 173, D-55216 Ingelheim am Rhein, Germany, is assignee of the entire right, title and interest for the United States of America (as defined in 35 U.S.C. § 100), by reason of an Assignment to the Assignee executed on (1) January 25, 1999 (2) February 1, 1999 (3) January 18, 1999 (4) January 19, 1999 (5) January 18, 1999 (6) January 18, 1999 (7) January 21, 1999 (8) January 18, 1999 (9) January 18, 1999 (10) January 18, 1999 (11) January 18, 1999 of an invention known as Process for Comparative Screening of Substances Having a Pharmacological Activity (Attorney Docket No. 0652.1830000/RWE/BEC), which is disclosed and claimed in a patent application of the same title by the inventor(s) (1) Armin Peter CZERNILOFSKY, (2) Thomas VON RÜDEN, (3) Adolf HIMMLER, (4) Gerhard LOEBER, (5) Thomas METZ, (6) Renate SCHNITZER, (7) Walter SPEVAK, (8) Christian STRATOWA, (9) Ulrike TONTSCH, (10) Ulrike WEYER-CZERNILOFSKY, and (11) Maria Josefa WICHE-CASTANON (said application filed on (International Filing Date: 25 June 1997) at the U.S. Patent and Trademark Office, having Application Number (U.S. National Phase of PCT/EP97/03329)).

The Assignee hereby appoints the following U.S. attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U S Patent and Trademark Office connected therewith: Robert Greene Sterne, Registration No. 28,912; Edward J. Kessler, Registration No. 25,688; Jorge A. Goldstein, Registration No. 29,021; Samuel L. Fox, Registration No. 30,353; David K.S. Cornwell, Registration No. 31,944; Robert W. Esmond, Registration No. 32,893; Tracy-Gene G. Durkin, Registration No. 32,831; Michele A. Cimbala, Registration No. 33,851; Michael B. Ray, Registration No. 33,997; Robert E. Sokohl, Registration No. 36,013; Eric K. Steffe, Registration No. 36,688, and Michael Q. Lee, Registration No. 35,239. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U S Patent and Trademark Office.

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DATE: January 19<sup>th</sup>, 1999

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My residence, post office address and citizenship are as stated below next to my name.

☒ was filed on 25 June 1997;  
as United States Application Number or PCT International Application Number PCT/EP97/03329; and  
was amended on December 23, 1998 (if applicable).

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

Analysis of Variance					
Source	Sum of Squares				
Corrected Total	10.000				
Between Groups	0.000				
Within Groups	10.000				
Total	10.000				
ANOVA					
Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Total	10.000	9	1.111		
Between Groups	0.000	1	0.000	0.000	1.000
Within Groups	10.000	8	1.250		
Total	10.000	9			
Descriptives					
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Between Groups	0.000	1	0.000	0.000	1.000
Within Groups	10.000	8	1.250		
Total	10.000	9			
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Corrected Total	10.000	9	1.111		
Between Groups	0.000	1	0.000	0.000	1.000
Within Groups	10.000	8	1.250		
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Total	10.000	9			

Prior Foreign Application(s)			Priority	Claimed
<u>96110459.3</u>	<u>Europe</u>	<u>28 June 1996</u>	<input checked="" type="checkbox"/> Yes	<input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)		
<u>                    </u>	<u>                    </u>	<u>                    </u>	<input type="checkbox"/> Yes	<input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application No.) \_\_\_\_\_ (Filing Date) \_\_\_\_\_

(Application No.) \_\_\_\_\_ (Filing Date) \_\_\_\_\_

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
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(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
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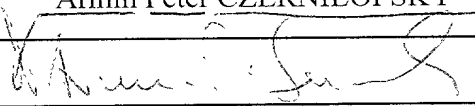
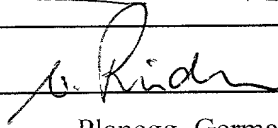
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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